THE
BIOLOGICAL BULLETIN

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VOLUME LXX
FEBRUARY TO JUNE, 1936

Printed and Issued by
LANCASTER PRESS, Inc.
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LANCASTER PRESS, INC., LANCASTER, PA.

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Volume LXX

Number 1

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LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Mass., between June 1 and October 1 and to the Institute of Biology, Divinity Avenue, Cambridge, Mass., during the remainder of the year.

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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

Vol. LXX, No. 1 February, 1936

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

INTEGUMENTARY COLOR CHANGES IN THE NEWLY-BORN DOGFISH, MUSTELUS CANIS

G. H. PARKER

(*From the Biological Laboratories, Harvard University*)

Introduction

It is well-known that the integumentary melanophores of the lower vertebrates are functionally active at a very early stage. These color-cells become responsive at about the time young fishes hatch from their eggs. This is true of *Fundulus*, according to Bancroft (1912), Spaeth (1913), and Gilson (1926) and of *Coregonus* and of two species of *Salmo* according to Becher (1929). At this stage the melanophores begin to disperse or concentrate their pigment, thus giving to the young fish a dark or a light tint. Although the beginnings of larval life may be said thus to mark the period of the first color changes, Becher noted that the embryos of fishes on which he worked when artificially removed from their egg-shells would often show color changes. This demonstrated that melanophore activity is possible at least under experimental conditions earlier than the time of hatching. Such a view was also taken by Duspiva (1931), who worked upon two fishes, *Salmo salvelinus* and *Perca fluviatilis*. In *Perca* the first integumentary melanin appears five days after the eggs have been fertilized. Duspiva saw melanophore responses three days later or about eight days after fertilization. He believed, however, that responses may have occurred still earlier. As the young larvæ of *Perca* hatch from the eggs about ten days after fertilization, the first melanophore responses in this fish must occur in what is obviously its embryonic period. From all his observations Duspiva was led to conclude that melanophores probably become active as soon as their processes are developed and their melanin formed. Such a view, which appears to be fairly well supported, places the initiation of melanophore activity in fishes at a very early stage, not later than about the time of hatching and probably in some species somewhat earlier.

The first responses of melanophores in lower vertebrates were shown by Babák (1910) to be quite unlike those of later life. In the

very young larvæ of the Mexican axolotl about 1.5 cm. long, when the first color changes had begun, the following conditions were found by Babák. In complete darkness the young animals were pale and in bright light they were dark. When they had attained a length of about 5 cm. their reactions in these respects were almost reversed. At this later stage they were in complete darkness dark and in bright light either pale or dark depending upon their surroundings. This second stage of their melanophore responses persisted until maturity when they became dark and remained permanently so, having lost their capacity to change. The early larval condition may be called the primary phase, the succeeding one the secondary phase. If a larva in the secondary phase is blinded, it was found by Babák to revert to the primary phase in that it would be pale in darkness and dark in bright light. A young larva in the primary phase when deprived of its eyes remained unchanged and was responsive in the same way as it had been before the operation. From these tests Babák concluded that what has been called here the primary phase was dependent upon the direct stimulation of the larval melanophores by light or its absence and that the secondary phase resulted from their indirect stimulation through the eyes of the larva. These conclusions were supported in one way or another by Pernitzsch's work (1913) on axolotl, by Hooker's work (1914) on *Rana pipiens*, and especially by an extended series of contributions from Laurens (1914, 1915, 1916, 1917) on the larvæ of several species of *Amblystoma*. Babák's views also received confirmation from the work of Fischel (1920) on various amphibians and especially from that of Duspiva (1931) on the two fishes already mentioned, *Salmo salvelinus* and *Perca fluviatilis*.

The references thus far cited pertain exclusively to oviparous fishes and amphibians. I know of only one writer who has recorded notes on the melanophore system of ovoviviparous vertebrates. In his paper on the genesis of chromatophores in fishes Eigenmann (1891) refers very briefly to the condition in the young of the ovoviviparous rockfish, *Sebastodes*, an embryo of which is figured by him. This small fish, when about to escape from the ovarian apparatus of the mother, is said by this author to have a very fully formed melanophore system whose functional possibilities, however, are not reported. Aside from this very meagre reference I know of no other on the melanophores in ovoviviparous vertebrates. It is therefore of interest to look into the condition of the newly-born pups of the smooth dogfish, *Mustelus canis* (Mitchill). This fish with its young can be had from time to time in the early summer at the Marine Biological Laboratory, Woods Hole, and I am under obligations to this Laboratory for the

material on which the following observations were made. I am also indebted to the Woods Hole Oceanographic Institution for the use of their equipment in working on these fishes. My studies were made under a grant given me from the Milton Fund of Harvard University for the investigation of color changes in marine animals. To the administrators of this Fund I wish to express my sincere thanks for the aid generously extended to me.

Observations

The smooth dogfish of the New England coast releases its young, four to a dozen or more at a time, in early summer. The following observations were made either on young dogfishes born in the laboratory tanks or on individuals removed from the females by what may be called a Cæsarean operation. After the envelopes and umbilical vessels of these young fishes had been severed they could be removed and carried gently to the sea-water tanks. During this procedure they were as a rule perfectly passive. After they had been immersed in the sea water of the tank for a fraction of a minute or so, normal respiratory gill movements began and at about the same time swimming was initiated. Such newly-born fishes swam for a short time with somewhat unsteady equilibrium, but they soon gained in steadiness and mingled with others of their kind in the tank. At the time of these tests in June the young fishes were about 25 cm. in length, the largest measuring 33 cm.

A young dogfish when first taken from the uterus of the female, a dark situation, is slightly dark in tint. This indicates that the young fish is in what has been called in this paper the secondary phase of melanophore activity, that is, the phase in which the fish is dark in darkness and pale or dark in the light according to the environment. Whether the dogfish passes through an earlier primary phase while it is still in the uterus of the mother is unknown. More likely this stage has been omitted in this fish as it appears to have been in the amphibians *Bombinator* and *Hyla* (Babák, 1910) and in the fish *Fundulus* (Bancroft, 1912; Spaeth, 1913; Wyman, 1924; Gilson, 1926).

Newly-born dogfishes respond in tint very quickly to that of their environment. On June 4 seven dogfishes were born in one of the experimental tanks. Five hours after their birth four were put in a white-walled, illuminated tank and three in a similar black-walled one. Two hours later those in the black-walled tank were extremely dark and those in the white-walled one decidedly light (Fig. 2). Preparations were made of the skin from an individual in each of these sets and microscopic views of the two preparations are shown in Figs. 3 and

4. As might be expected, the melanin in the color-cells from the pale dogfish is concentrated (Fig. 3), that from the dark one is dispersed (Fig. 4). On the following day the pale fishes were put into the black-walled tank and the dark ones into the white-walled one. Within two hours the fishes had reversed their tints, the pale ones having become dark and the dark ones pale.

In a second litter of dogfishes obtained by Cæsarean operation three were put immediately after their removal from the uterus into a black-walled illuminated tank and three into a white-walled one. The three fishes destined for the black tank were put into it at 9:17 in the morning. By 9:55 they had increased their dark tint. At 10:30 they were dark but not fully so, and at 10:50 they were fully dark. The three dogfishes intended for the white tank were put into it at 9:20 in the morning. By 9:55 they had lightened considerably but were still somewhat dark. By 10:30 they were fully blanched as compared with other young dogfishes used as checks. From these two sets of tests it is fair to conclude that specimens of *Mustelus* immediately after birth are capable of responding to the tint of their environment by appropriate melanophore reactions. In these responses they were quite like adults of their own species.

In another respect the young dogfishes also resembled adults. It has already been shown (Parker and Porter, 1934) that when a small transverse cut is made in the fin of a relatively dark *Mustelus* a pale band is soon formed extending from the cut toward the free edge of the fin. This band is believed to result from an excessive stimulation of the concentrating melanophoric nerve-fibers cut by the operation. Similar conditions obtain in the newly-born dogfishes. The pectoral fins of two such fishes were cut as described and 25 minutes after the operation both fishes had well-developed pectoral bands. One of these fishes was killed and a preparation of its pectoral fin was made. A photograph of this fin is reproduced in Fig. 1 and shows the typical pectoral bands.

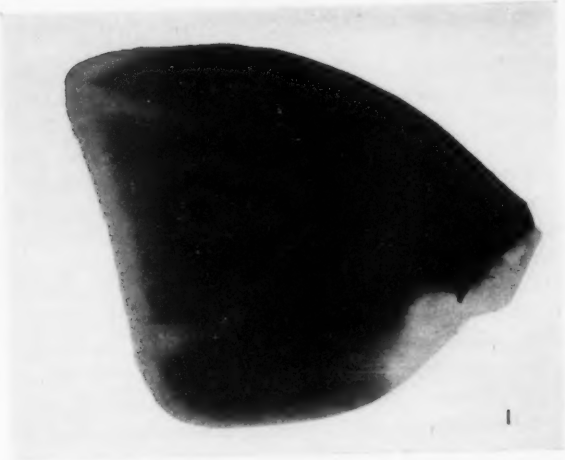
EXPLANATION OF PLATE

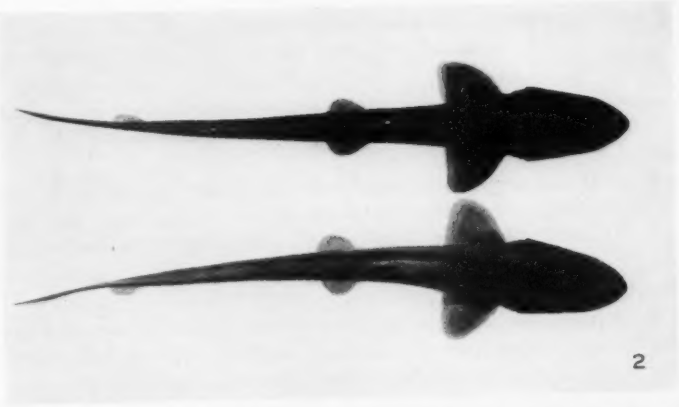
FIG. 1. Dorsal view of the left pectoral fin of a newly-born *Mustelus canis*. Two small cuts transverse to the fin rays have been made, the anterior one nearer the root of the fin, the posterior one nearer its lateral edge. A pale band extends from near each cut to the light edge of the fin.

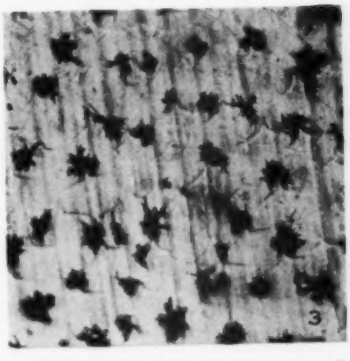
FIG. 2. Dorsal views of two newly-born *Mustelus canis*, the upper one in the dark condition, the lower one in the light condition. Preparations preserved in formaldehyde-alcohol.

FIG. 3. Microscopic view of the melanophores on the dorsal surface of the pectoral fin of a *Mustelus canis* in the light condition, melanin concentrated.

FIG. 4. A view similar to that shown in Fig. 3 of the fin of *Mustelus canis* in the dark condition, melanin dispersed.







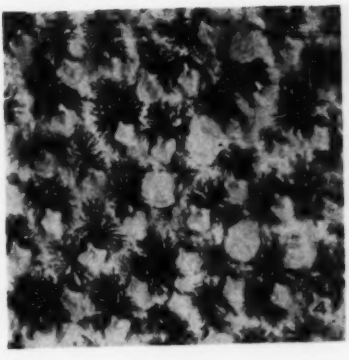


PLATE I

In a third respect newly-born dogfishes resemble adults. They respond in the same way to adrenalin and to pituitrin (Parke, Davis and Company's preparations) as the mature dogfishes do (Lundstrom and Bard, 1932). If 0.2 cc. of a solution of adrenalin, one part in a thousand of water, is injected into a young dogfish of moderately dark tint, the animal will begin to blanch in about ten minutes and shortly after that it will assume a tint of extreme paleness. After two to three hours the young fish will reassume its darker tone. In a similar way an injection of 0.2 cc. of obstetrical pituitrin into a pale dogfish will induce its slow darkening. This change also passes away in about two hours after which the young dogfish will return to its former state. In both these respects the newly-born fishes resemble the adult.

These records show quite clearly that a young *Mustelus* immediately after birth possesses an active melanophore system whose reactions in a number of significant ways agree very fully with those of the adults. These ovoviviparous dogfishes then begin life in what has been called the secondary phase of melanophore activity. On the loss of their eyes they should lapse, according to the general theory of these relations, to the condition of the primary phase. Unfortunately my material was not sufficiently abundant to allow this test to be made.

Summary

1. *Mustelus canis* is an ovoviviparous dogfish in which the young are born with a body length up to 33 cm.

2. At birth the young dogfishes are of a moderately dark melanophoric tint. This is doubtless the influence of the maternal body within which they have been lodged.

3. Immediately after birth these young dogfishes respond to their environment in that they change light or dark, conditions brought on by a concentration or a dispersion of their melanophore pigment.

4. Pale bands can be produced on the fins of newly-born *Mustelus* by cutting their nerves, as can be done with the adults.

5. A young *Mustelus* responds to injections of adrenalin by blanching and to pituitrin by darkening as adults do.

6. A newly-born *Mustelus* shows no evidence of the primary phase of color change seen in some other fishes and in some amphibians. It appears to omit this phase in its ontogeny and is born with a melanophore system that responds in the same way as this system does in the adults.

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LIGHT INTENSITY AND MELANOPHORE RESPONSE IN THE MINNOW, ERICYMBA BUCCATA COPE

F. A. BROWN, JR.

(From the Zoölogical Laboratory of the University of Illinois) [1]

Although every investigator of chromatic response in animals is well aware that light intensity has a profound effect upon the coloration of animals, this knowledge is at most qualitative and is in general restricted to the effects of the presence or absence of light. Fishes become pale in darkness as a rule while in light the response is governed by the color of the background. Considering that many animals with responsive pigmentary systems are normally accustomed to live at relatively low intensities of light, it was believed of significant value to investigate the behavior of fish from this viewpoint, and perhaps to derive simultaneously more information in regard to the sensitivity of the photoreceptors and the manner in which they function to produce adaptive color changes. In this latter regard Keeble and Gamble (1904), Bauer (1905), Sumner (1911), von Frisch (1911), Sumner and Keys (1929), Pearson (1930), and Sumner (1933) have concluded that the color of the animal is determined by the ratio of incident and reflected light striking the eye of the animal. Should this statement hold true without modification, then it would follow that for a given background which is characterized by always reflecting a given percentage of the light striking it, the animal would remain the same shade at all effective light intensities and there would exist a sharp critical point in intensity of light at which a fish upon a black background would become pale as the light was decreased. Whether or not this is the actual state of affairs is tested in the following experiment.

I. Uniform Black Background and Varying Light Intensity

Material and Methods

In the experiment herein described the silver-mouthed minnow, *Ericymba buccata* Cope, was used exclusively. This fish is from five to eight centimeters in length and is found most abundantly in streams with sand bottoms in the region about Urbana, Illinois, (Thompson and Hunt, 1930). The fish used were always freshly caught, none

[1] Contribution No. 474.

being used after they had been in the laboratory tanks for more than a week. Furthermore, all the data were obtained from fish caught in a given region and within a period of less than two winter months, thus insuring fish that were quite uniformly pigmented. Because of the rapidity of color change in this species no method was devised whereby more than a single light intensity could be used in testing the quantitative response in any individual fish. As a result, the data included here are those of a population and hence characterized by considerable variation.

All the experiments were carried out in an experimental dark-room. The light sources used in obtaining the different intensities were a 200 watt tungsten lamp, a 75 watt daylight Mazda lamp, and a 25 watt tungsten lamp with a daylight filter. For the lower intensities the last lamp was enclosed in a light-proof container possessing an iris diaphragm over the single aperture. In addition, approximately neutral light filters (exposed photographic plates) were used. The measurement of the light intensity was made with a potassium photoelectric cell with maximum sensitivity in the blue end of the visible spectrum. This cell was constructed by Professor J. Kunz. In nearly every instance the light source was 125 centimeters directly above the 8 inch, flat-black-coated crystallizing dish in which the experimental fish were placed.

In order to test the effect of a given light intensity upon the response of the melanophores, two or three minnows were placed in the crystallizing dish containing three centimeters of water. The crystallizing dish was coated with flat-black paint because it was determined that upon a background of this nature the fish would become dark in color in the ordinary light intensities of the laboratory and would become pale in total darkness. Thus the actual intensity at which the fish would entirely cease to respond to the color of the background could be learned. At the end of two to three hours in the experimental situation, during which time the water in which the fish were kept was constantly aerated with compressed air and an aerator block, the fish were removed and immersed in boiling water for a minute or two. Then a piece of the integument from a given area of the fish was dissected off and mounted in glycerine. A Spencer camera lucida and a celluloid rule served in measuring the diameters of 30 to 80 melanin masses. The average was taken as an index of the degree of dispersion of the pigment in the fish as a whole. In order to ascertain that two hours was sufficiently long for the pigment masses to have reached an equilibrium point of dispersion within the melanophores for the light intensity and background in question, some of the fish were taken from

a black background in light and others from a white background in light at the beginning of the experiment and thus the equilibrium points were reached from both sides. Furthermore, at the intensities of the ordinary daylight of the laboratory the color-change was very rapid, requiring only five or ten minutes to pass from one extreme to the other. Research is now in progress to determine to what extent the rates of color change are influenced by intensity.

Results

During the course of the experimentation 75 minnows were tested. These were roughly distributed among the seven light intensities in

TABLE I

Average diameters of melanin masses in fish kept upon a black background under measured light intensities. Pigment mass diameters are given in micra and light intensity in foot candles.

Light intensity						
.000005	.000053	.000625	.0125	.25	1.75	23.75
55.3	65.9	78.8	78.6	80.5	86.1	88.5
52.5	48.4	68.3	76.9	78.5	84.4	82.0
40.5	43.7	63.4	74.0	76.3	83.4	80.3
40.4	41.6	58.3	72.5	64.3	81.4	80.2
36.6	38.2	56.4	68.6	54.0	80.5	79.8
33.9	39.3	55.0	67.6	74.5	77.1	68.6
	36.3	51.9	59.6	73.6	77.0	66.7
	34.3	51.1	63.5	72.5	76.6	
	34.0	44.0	54.3	69.1	75.3	
	40.7	41.9	53.5	59.7	74.7	
	41.4	38.9	51.3	57.5	64.3	
		34.0	49.3	54.6		
			46.9			
			46.3			
			45.6			
			36.6			
Mean 43.1	43.4	53.4	59.2	67.9	78.3	78.0

proportion to the variability exhibited in the data. Table I is a summary of the results.

The averages for a number of fish have been calculated for each light intensity. The values for individual fish are shown diagrammatically in Fig. 1, plotted against the logarithm of the light intensity. These data strongly suggest that the relationship is most probably one of a direct proportionality over the complete range of dispersion of pigment mechanically possible in the melanophore of this fish. In

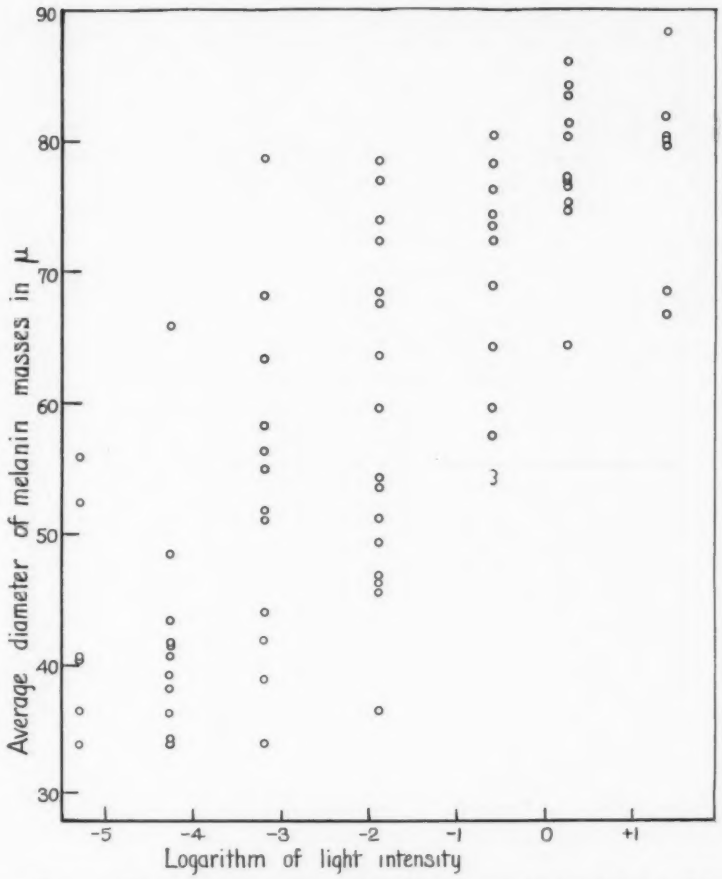

FIG. 1. Diagram showing the relationship between the logarithm of light intensity and the degree of melanin dispersion in the melanophores of *Ericymba buccata*. Each dot indicates the value for a single fish.

other words, the limits of this simple relationship are determined by the diameters of completely concentrated and fully dispersed pigment masses.

II. UNIFORM LIGHT SOURCE WITH DIFFERENT SHADES OF GRAY BACKGROUNDS

Material and Methods

A second type of experiment was performed using the same material and methods as in the first, except that here the light was kept constant

at 1.75 foot candles, the minimum intensity at which complete dispersion of melanin could be obtained in response to a black background. Six backgrounds were used, the black one of the previous experiment, a white one, and four of intermediate shades of gray. The latter five were obtained by placing a glass crystallizing dish upon paper and cloth of the appropriate shades. The relative brightnesses of the direct light at the level of the background and the light reflected from the background were measured with a Macbeth illuminometer equipped with a brightness-determining device consisting of a trans-

TABLE II

Average diameters of melanin masses in fish kept upon black, white, and gray backgrounds in an incident light intensity of 1.75 foot candles. The ratio indicates the $\frac{\text{incident intensity}}{\text{reflected intensity}}$. Pigment mass diameter is given in micra.

Background ratio					
2.54	6.60	12.4	35.4	140	201
37.2	38.2	35.6	56.0	75.7	86.1
31.6	39.2	35.0	45.3	73.6	84.4
35.7	36.2	40.0	42.7	70.7	83.4
37.9			40.9	68.0	81.4
40.3			40.7	63.0	80.5
32.3			41.4	57.1	77.1
			46.4	55.7	77.0
			74.5	77.8	76.6
			59.5	74.8	75.3
			37.3		74.7
			38.2		64.3
			46.7		
			48.6		
Mean 35.8	37.9	36.9	47.5	68.6	78.3

lucent white plate. The values obtained serve best as comparative ones. The value of the ratio, $\frac{\text{direct light}}{\text{reflected light}}$, obtained for the white background was 2.54; for the gray backgrounds, 6.60, 12.4, 35.4, and 140.; and for the black one, 201.

Results

The average diameters of the melanin masses in 45 fish kept upon these above-described backgrounds are recorded in Table II. The values for the black background have been taken from Part I of this paper. These results plotted as a dot diagram in Fig. 2 indicate that

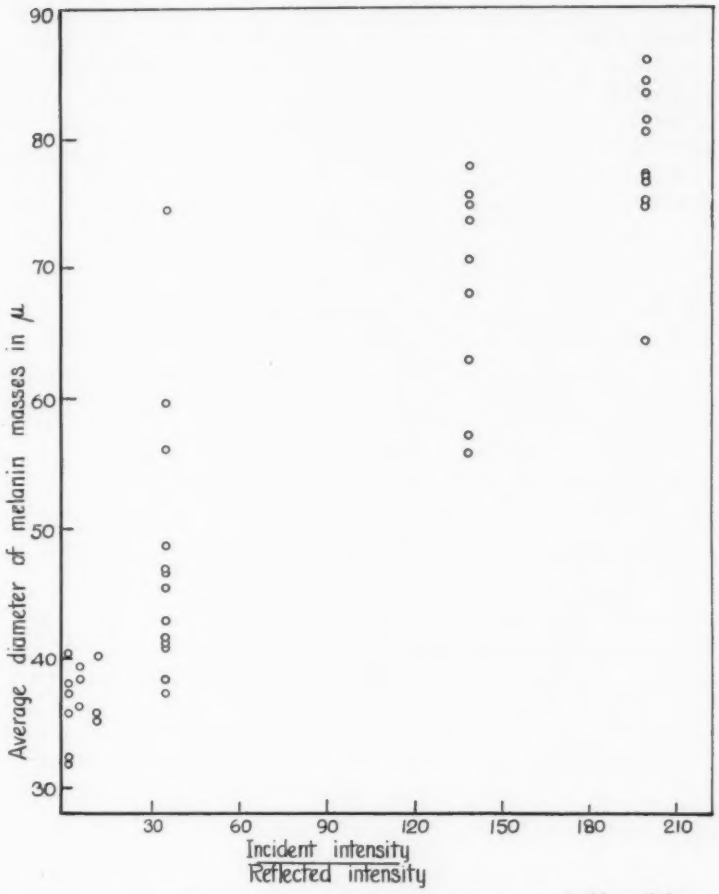

FIG. 2. Diagram showing the relationship between the ratio, $\frac{\text{incident light}}{\text{reflected light}}$, and the degree of melanin dispersion in the minnow, *Ericymba buccata*. Each dot indicates the value for a single fish.

for a given light intensity the average diameter of the pigment within the melanophore is a direct function of the ratio of incident to reflected light.

GENERAL CONSIDERATIONS

The ratio of incident to reflected light is thus seen to be inadequate to account completely for the degree of melanophore dispersion even

when the intensity is considerably above the value of zero. Total intensity has a marked and significant effect. Duspiva (1931) found that light intensity had a very great effect upon the coloration of larvæ of *Perca fluviatilis*, *Salmo salvelinus*, and some other fishes. Background was without effect upon these forms. Koller (1934), however, reported that for larval *Coregonus* the background was the principal stimulus for melanophore activity while the light intensity had no influence until complete darkness was attained. These two papers are characteristic of the extremes in the literature regarding the effect of light intensity upon melanophores in fishes. The present research does not pretend to give a complete answer to this problem of long standing. Rather, it merely suggests that both forces are quite definitely influential in altering the coloration of fishes, but that the degree of overlapping of the two or the dominance of a single one determines the differences between individual species and even the same species at different developmental periods.

It is a well-known fact to fishermen of the Illinois River that when the water is laden with silt and consequently permits light to penetrate only a very short distance in any quantity, the fishes taken are pale in color, whereas in clear water the fishes are invariably dark in shade. If fishes swimming freely at some distance from the bottom in clear water can be figured as being upon the equivalent of a black background since practically no light is reflected from below, then the silt in the more turbid waters can be conceived of as having effect through reduction of incident light and also augmentation of the reflected light entering the eyes of the fishes.

In closing, I wish to acknowledge my indebtedness to Dr. David H. Thompson of the Illinois Natural History Survey and to Professor J. Kunz of the Physics Department, University of Illinois, for generously supplying me with helpful suggestions and material during this investigation.

Summary

1. A quantitative method of determining the influence of the environment upon the melanophores of small fishes is described.

2. The degree of dispersion of the melanin in the melanophores of the silver-mouthed minnow, *Ericymba buccata*, is within certain limits determined by the total light intensity as well as by the shade of the background.

3. Upon a constant black background between the intensities of light, .000053 and 1.75 foot candles, the average diameter of pigment masses is directly proportional to the logarithm of the light intensity.

4. At an intensity of less than .000053 foot candles the fish are at their maximum degree of paleness in spite of a black background.

5. At 1.75 foot candles illumination the fish becomes maximally dark upon a black background, and the average diameter of melanin masses appears to vary in a directly proportional fashion with the ratio, $\frac{\text{incident light}}{\text{reflected light}}$, which reaches the eyes of the fish.

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RENAL FUNCTION IN MARINE TELEOSTS

II. The Nitrogenous Constituents of the Urine of Sculpin and Flounder, with Particular Reference to Trimethylamine Oxide

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In view of the rather extensive work which has been done upon kidney function in the sculpin (*Myoxocephalus octodecimspinosus*), a common marine teleost (for review of literature see 34), it became of interest to investigate more fully the nitrogenous constituents of the urine. For comparison the flounder (*Pseudopleuronectes americanus*) was likewise studied. Analysis of samples of urine from these two species for the ordinary nitrogenous constituents showed that approximately one-half of the total nitrogen was still unaccounted for. The presence of trimethylamine oxide in other marine fishes (see Table III) made it appear likely that this compound might be present in the urine of our animals. Investigation has demonstrated its occurrence in sculpin urine in significant amounts, but its absence in detectable quantities from the urine of the flounder. Brief supplemental studies demonstrated the occurrence of the oxide in the urine of the daddy sculpin (*Myoxocephalus scorpius*) and confirmed its presence in the urine of the goosefish (*Lophius piscatorius*) (18).

Material and Methods

Freshly caught sculpins and flounders were brought into the laboratory, the urinary papilla was tied off, and the fish were transferred to live cars, usually for 24 hours, sometimes longer. At the end of this time they were killed by a blow on the head, and the urine was removed from the exposed bladder by syringe. Following are additional details concerning the urine samples listed in Tables I and II; unless otherwise noted preservation was by toluol and hydrochloric acid. All samples were kept in a refrigerator when not in use. *Sculpin 1:* about 475 cc., from 217 sculpins; chloroform and toluol. *Sculpin 2:* about 475 cc., from 239 sculpins; 1 per cent sulfuric acid. *Sculpin 3:* 15 cc. from a few specimens. *Flounder 1:* about 475 cc., from 135 flounders; chloroform and toluol. *Flounder 2:* about 475 cc.,

[1] With the assistance of Gordon Spence.

from 117 flounders; 1 per cent sulfuric acid. *Flounder 3:* 15 cc. from a few specimens. *Flounder 4:* same. *Daddy sculpin:* from two specimens. *Goosefish:* from one specimen in good condition. All sculpins and all but one group of fifty flounders (taken in net) were caught with hook and line. The urine samples were collected at the Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine.

Total nitrogen was determined by the methods of Van Slyke (49) (*Sculpin 1* and *2*, *Flounder 1* and *2*) and Pregl (39) (all other specimens); ammonia by the method of Folin and Bell (13); urea by the method of Van Slyke (50), using 0.5 M. veronal rather than phosphate as a buffer because of the high magnesium content of the urine; uric acid by the method of Benedict and Franke (4); amino acids by the method of Folin (12); total creatinine by the method of Folin (11); and chloride by the method of Van Slyke (48) as modified by Smith (41). Trimethylamine oxide was determined by the method of Hoppe-Seyler (24).

Results

The analytical data for the ordinary nitrogenous constituents of the large pooled samples of sculpin and flounder urine are summarized in Table I. The chloride values for these samples, in millimols per

TABLE I

Distribution of nitrogenous constituents of sculpin and flounder urine.

	Sculpin 1		Sculpin 2		Flounder 1		Flounder 2	
	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen
	mgm./100 cc.	*per cent*	*mgm./100 cc.*	*per cent*	*mgm./100 cc.*	*per cent*	*mgm./100 cc.*	*per cent*
Total N	88.0		84.5		43.4		84.0	
Urea N	13.5	15.3	11.9	14.1	9.2	21.2	11.3	13.5
Ammonia N	1.1	1.3	1.7	2.0	0.8	1.8	2.2	2.6
Uric acid N	0.6	0.7	0.6	0.7	0.5	1.2	1.1	1.3
Amino acid N	3.9	4.4	3.4	4.0	4.2	9.7	6.9	8.2
Total creatinine N	19.7	22.4	21.1	25.0	6.5	15.0	21.9	26.1
Undetermined N	49.2	55.9	45.8	54.2	22.2	51.1	40.6	48.3

liter, are as follows: *Sculpin 1*, 99; *Sculpin 2*, 105; *Flounder 1*, 184; *Flounder 2*, 138. It has previously been demonstrated (15) that sculpins, when handled under rather ideal conditions, with marked precautions to avoid skin injury, regularly show a higher average urinary total nitrogen and a lower average urinary chloride (see also 16) than those reported here. With repeated handling under the usual experimental conditions, the animals typically develop a diuresis, which is characterized by a rather rapid decrease in urinary con-

centration of total nitrogen and a sharp increase in the urinary concentration of chloride (15, 37). Under the circumstances, the divergence in the present samples from the more ideal values can undoubtedly be attributed to the manner in which the sculpins were handled. With the large number of animals used, the handling was necessarily rather rough as compared to the rigid technic employed in more exacting experiments (15, 16). This also applies to the total nitrogen value given for the daddy sculpin (Table II). From urinary chloride data upon flounder presented elsewhere (16), the high chloride and low total nitrogen values in the samples of flounder urine can be similarly interpreted. In line with this interpretation is the fact that specimen *Flounder 1*, containing urine from a batch of fifty flounders subjected to rather severe conditions and overcrowding in a flounder net, shows a very low total nitrogen and a very high chloride concentration. The total nitrogen content of the sample of goosefish urine (Table II) is well below the values reported by Grollman (18) for bladder urine from freshly caught specimens, is comparable with those given by Edwards and Condorelli (8), and is far above those reported by Marshall and Grafflin (35) and Smith (40), whose specimens were obviously quite diuretic.

The abnormalities in urine formation observed under experimental conditions are associated primarily with a derangement of the normal salt and water balance of the organism, resulting from the loss of water through the skin and the increased ingestion and gastro-intestinal absorption of sea water (42, 17). The important fact, for the present data, is that from all available evidence there is no concomitant derangement of nitrogen metabolism under these conditions; i.e., the decreased urinary concentration of total nitrogen represents primarily a dilution effect. This statement is borne out by a comparison of the nitrogen distribution data upon the urine of the goosefish previously reported by Grollman (18) and Smith (40), the former at high and the latter at low urinary total nitrogen concentration. Under the circumstances, the analytical data given in Table I are interpreted as representing fairly accurately the normal percentage distribution of the ordinary nitrogenous constituents and percentage of undetermined nitrogen in sculpin and flounder urine.

The ammonia and uric acid values for sculpin and flounder are quite low, and are comparable with those reported by Grollman (18) for *Lophius*, but much lower than those reported for ammonia in *Lophius* and *Muræna* by Edwards and Condorelli (8). Urea is present in considerable amounts in sculpin and flounder urine, some data on sculpin having been reported by Marshall and Grafflin (36). The

urinary excretion of urea in significant amounts seems to be characteristic of all marine teleosts so far studied except the goosefish (*Lophius piscatorius*). Marshall and Grafflin (35) reported that the plasma and urine of this species uniformly contain urea in only minimal amounts, and this was subsequently confirmed for the urine by Grollman (18) and Smith (40). The high values reported for *Lophius* by Edwards and Condorelli (8) seem to cast doubt upon all of their urea analyses. The amino-acid and total creatinine values for sculpin and flounder are to be compared with other data available in the literature (8, 18, 35, 37, 40). In the marine teleosts as a group, the total creatinine fraction of the urine consists predominantly of creatine. Fairly extensive data upon the creatine content of sculpin urine, under normal and diuretic conditions, have been supplied by Pitts (37).

It is evident from Table I that approximately one-half of the total nitrogen in the composite samples of sculpin and flounder urine is unaccounted for by the ordinary nitrogenous constituents. The

TABLE II

Trimethylamine oxide in urine of sculpin, flounder, daddy sculpin and goosefish.

	Sculpin 3	Flounder 3	Flounder 4	Daddy sculpin	Goose-fish
Total N, *mgm. per cent*	98.5	66.2	65.8	44.5	212.0
$(CH_3)_3NO$ N, *mgm. per cent*	22.8	−0.3	1.4	14.1	69.1
Total N, *per cent*	23.1	—	—	31.7	32.6

demonstrated presence of trimethylamine oxide—$(CH_3)_3NO$—in the muscles of marine elasmobranchs and teleosts and particularly its presence in the urine of the goosefish (see Table III) made it appear likely that this compound might be present in the urine of our animals. Using the micro-method of Hoppe-Seyler (24), results were obtained which indicated the presence of the oxide in considerable amounts in sculpin urine, but its absence in readily detectable quantities from flounder urine. Since there was a rather long interval between the collection of these urine samples and their analysis for trimethylamine oxide, and since the oxide has been found to undergo a slow decomposition to trimethylamine even in well-preserved urine (18), these data were rejected and new urine samples were collected in the following summer. These samples were analyzed soon after collection (Table II) and confirmed the previous findings. The $(CH_3)_3NO$ figure (22.8) for the fresh composite sample of sculpin urine (*Sculpin 3*)

is to be compared with the figure 20.4 (milligrams per cent of nitrogen as $(CH_3)_3NO$) obtained upon sample *Sculpin 2* (preserved with sulfuric acid) more than two months after collection. This latter value represents 24.1 per cent of the total nitrogen, 44.5 per cent of the undetermined nitrogen. Although present in sculpin urine in considerable amounts, trimethylamine oxide is apparently entirely absent from flounder urine, or present in only small traces. The amount present in the urine of the daddy sculpin is comparable with that in the sculpin; and the value for goosefish urine is essentially confirmatory of the results of Grollman (18).

In order to obtain additional evidence that the substance present in sculpin urine is actually trimethylamine oxide, this compound was isolated from urine sample *Sculpin 1* as the aurichloride. For trimethylamine oxide this salt would have the composition $(CH_3)_3NO \cdot HAuCl_4$. Synthetic material of this composition gave a melting point of 255–7°, which agreed exactly with that of the salt isolated from sculpin urine. The mixed melting point was likewise 255–7°. On analysis the sculpin salt gave the percentage of gold as 46.0, 46.25 (theoretical 47.5 per cent); percentage of nitrogen 3.5 (theoretical 3.4 per cent). From the aurichloride the picrate was prepared in the following manner. The gold salt was dissolved in water and treated with "molecular" silver until the yellow solution became colorless. The silver was then centrifuged off and slightly less than the calculated amount of picric acid solution was added. The melting point of the picrate from the sculpin gold salt and of synthetic picrate from trimethylamine oxide, as well as the mixed melting point, all agreed: 194–7°. In addition, the picrates from the natural and synthetic sources had the same crystalline form.

Thus there is adequate evidence that trimethylamine oxide makes up a considerable portion of the "undetermined" nitrogen fraction of sculpin urine. As to the nature of the compounds present in the remaining fraction of the undetermined nitrogen in this species, we have no satisfactory data. This applies also to the entire undetermined nitrogen fraction of flounder urine.

Discussion

The presence of trimethylamine oxide and trimethylamine in the fishes has been the subject of considerable investigation, and the present state of our knowledge is summarized in Table III. Supplemental to the table is the clear demonstration of the presence of trimethylamine oxide in the blood of *Scyllium catulus* (24) and in the roe

of *Clupea harengus* (38).[2] It is obvious that our negative finding for the urine of the flounder, *Pseudopleuronectes americanus*, is particularly striking in view of the positive results obtained from the muscles or urine of all other marine teleosts so far examined, especially from the muscles of the closely related *Pleuronectes cynoglossus*. The generalization has frequently been made that trimethylamine oxide is characteristic of marine, as opposed to fresh water, teleosts. Its absence from the urine of the flounder does not, naturally, preclude the possibility of its occurrence in the muscles of this species.

TABLE III

The occurrence of trimethylamine oxide and trimethylamine in the muscles and urine of fishes.

	Muscles		Urine		References
	TrO	Tr	TrO	Tr	
Selachians (Marine)					
Acanthias vulgaris	+	+	+	−	21, 24, 43, 44
Lamna cornubica	+	+	−	−	21
Scyllium catulus	−	−	+	+	24
Teleosts					
a. Marine					
Clupea harengus	+	+	−	−	21, 38
Gadus æglefinus	+	+	−	−	21, 25
Gadus morrhua	+	+	−	−	21, 25, 26
Mullus barbatus	+	+	−	−	21, 25
Pleuronectes cynoglossus	+	+	−	−	21, 25
Pseudopleuronectes americanus	−	−	0	−	This paper
Conger vulgaris	+	+	−	−	21, 25
Lophius piscatorius	−	−	+	+	18, this paper
Myoxocephalus octodecimspinosus	−	−	+	−	This paper
Myoxocephalus scorpius	−	−	+	−	This paper
b. Fresh water					
Anguilla vulgaris	0	0	−	−	21, 25
Salmo salar	0	0	−	−	21, 25
Squalius cephalus	0	0	−	−	21, 25
Alburnus lucidus	0	0	−	−	21, 25
Perca fluviatilis	0	0	−	−	21, 25
Cyprinus carpio	0	0	0	0	21, 24

+ = present; 0 = absent; − = untested; TrO = trimethylamine oxide; Tr = trimethylamine.

[2] Grollman (18) concludes that the trimethylamine present in goosefish urine was probably absent from the fresh specimens, and resulted from the slow decomposition of the oxide; and Hoppe-Seyler (24) indicates the possibility of a similar origin for the amine present in his samples of selachian urine. He further states (23, 24) that one cannot for the present be certain that the small amounts of trimethylamine previously (21) found in extracts of fresh muscles of selachians and marine teleosts (and originally regarded as a metabolic rather than a decomposition product) do not arise from the oxide in the course of the working-up process. He could also detect no relationship between the presence of the amine and its oxide and seasonal variations or the spawning period.

Trimethylamine oxide has further been demonstrated in cephalopods (20) and, on the basis of Hoppe-Seyler's interpretation (22), in crustacea by Suzuki *et al.* (45, 46), who reported it as "kanirin."[3] From the gonads of the jellyfish, *Rhizostoma cuvieri*, Haurowitz (19) has isolated trimethylamine, which is accepted as existing preformed in the tissues (21). Neither the amine nor its oxide has ever been reported as occurring in the normal tissues or body fluids of amphibia, reptiles or birds. Kappeler-Adler and Krael (26) reported an indirect demonstration of the oxide in mammalian muscle, and Lintzel (32) has very recently claimed trimethylamine and trimethylamine oxide to be normal constituents of human urine.[4]

From a detailed consideration of the osmotic relationships existing between the fishes and their environment, Hoppe-Seyler (23, 24) concludes that the retention of nitrogenous metabolic products creates the conditions which are necessary for the formation of the oxide. Thus the selachians, which are able to hold the salt content of their body fluids much lower than that of sea water, establish isotonicity with their environment through the retention of urea, trimethylamine oxide and betaine, and thus hinder the withdrawal of water from the body by the surrounding medium. Actually, Hoppe-Seyler's conception of the existing relationships in marine elasmobranchs is erroneous. Selachian blood is hypertonic to sea water, and this group is to be classed along with fresh water fishes as osmotically superior to the environment (42). That there is an active conservation of the oxide by the kidneys has been established by his demonstration of the much lower concentration in the urine as compared with the blood. According to Hoppe-Seyler, in the marine teleosts a similar, but much smaller, retention of metabolic end-products must occur, and in this

[3] The closely related tetramethylammonium hydroxide has been isolated from *Actinia equina* (2).

[4] The presence of trimethylamine in human urine has often been reported (among others, 5, 10, 14); but following the investigations of Takeda (47), Kinoshita (27) and Erdmann (9) it has been generally accepted that the amine does not exist preformed in the urine, but is liberated from some precursor by either chemical or bacterial decomposition. Lintzel refuses to accept this conclusion, and goes on to show that when large quantities of trimethylamine oxide, betaine, choline, etc. are added to human urine, the yield of trimethylamine does not increase. He further reports that when urine is distilled for trimethylamine for several hours, a subsequent distillation yields no more of the substance, even though a considerable quantity of trimethylammonium base is present. Actually, Takeda concluded that preformed trimethylamine is perhaps present at times in normal human urine, though always absent from that of the dog and horse. Trimethylamine has likewise been reported in human cerebrospinal fluid (7) and in the blood of lower mammals (6, 7) and man (7, 14). However, these reports have all been subjected to the same criticism that the amine may well have arisen from some precursor through chemical or bacterial decomposition.

way the difference between the osmotic pressure of the sea water and of the blood is at least diminished. The validity of this argument has been questioned by Homer Smith, in an article to appear in *Biological Reviews*, on the ground that direct evidence for an active retention in marine teleosts has never been adduced. On the other hand, in fresh water teleosts, living in a markedly hypotonic medium, the conditions are such that metabolic products are withheld as little as possible, and the oxide never occurs (Table III).[5] Hoppe-Seyler extends his argument to the presence of the oxide in cephalopods and of the betaines in marine invertebrates and in plants, and makes the inclusive generalization that wherever nitrogenous metabolic products are retained, the preliminary conditions for the occurrence of methylation processes are present.

The conclusion of this investigator (21, 23), that on the basis of all available data trimethylamine and trimethylamine oxide must be considered primarily as end-products of protein metabolism, is concurred with by Poller and Linneweh (38) and Grollman (18), and is confirmed by the demonstration of the oxide in the urine of selachians and marine teleosts (23). This is in agreement with the general view expressed by Kutscher and Ackermann (29, 30) for the methylated muscle bases. Hoppe-Seyler (21, 23) regards trimethylamine and its oxide as derived primarily from ammonia through methylation and oxidation; and sees in the formation of the oxide the replacement of ammonia, which is toxic for the vertebrates in high concentration, by an essentially non-toxic, very weakly basic compound. The other methylated muscle bases, on the other hand, most probably are derived from higher degradation products of proteins, the amino acids (30). The idea that trimethylamine and its oxide may arise from the betaines, cholines (lecithin) and other compounds has also been advanced (18, 21, 28), and Lintzel (32) accepts such an origin in the human through the activity of the intestinal bacteria (see below).

The possibility that trimethylamine and its oxide are, in the fishes, in part at least of exogenous origin, being ingested preformed, has been

[5] Hoppe-Seyler (23) sought to settle the question whether trimethylamine and its oxide actually do appear as metabolic products in fresh water teleosts, but are not demonstrable because of their rapid and complete excretion in the dilute urine. Carp were allowed to swim about in limited quantities of water over a period of seven days, and the entire body of water (containing the urine), when concentrated to small volume and treated with zinc dust and HCl, gave no precipitate with gold chloride. The logical conclusion was that the amine and its oxide are not formed in fresh water fish. Smith (40) reported a volatile base, in addition to ammonia, in the branchial and urinary excretions of the carp, and concluded that it was presumably in large part trimethylamine liberated from the oxide on treatment with zinc dust and alkali. The inability of Hoppe-Seyler to detect either the oxide or the free amine in the urine or muscles of this species (21, 24) renders Smith's interpretation untenable.

accepted by Grollman (18) and Hoppe-Seyler (21). The latter, however, believes it impossible that this could form the only source for the amounts demonstrable in selachians and marine teleosts, even though one assumed a strong retention of nitrogenous metabolic products. Lintzel (32) similarly accepts an exogenous origin for part of the trimethylamine and trimethylamine oxide in human urine. According to the latter investigator, ingested trimethylamine would appear in the urine principally as the oxide.

Hoppe-Seyler (21) has concluded that one must consider the possibility that in the fishes trimethylamine and its oxide can, at least in part and under certain conditions, be demethylated. From the work of Löffler (33), who showed an increase in urea formation when the surviving liver was perfused with trimethylamine, it is reasonable to assume that demethylation is a normal process in the mammals.

Suwa (44) first showed that in the presence of putrefying tissue trimethylamine oxide was converted to the free amine; and Poller and Linneweh (38), on the basis of their demonstration of the oxide in the muscle of *Clupea harengus* and this known bacterial conversion, offered an explanation for the large amounts of trimethylamine known to be present in herring brine. Ackermann and Schütze (1) and Kohlrausch (28) demonstrated the liberation of the amine from lecithin, choline and betaine under bacterial influence; and Takeda (47) demonstrated the appearance of trimethylamine in considerable amounts in mammalian urine allowed to undergo ammoniacal fermentation. Later, Ackermann, Poller and Linneweh (3) showed that the conversion of trimethylamine oxide to the amine, demonstrated by Suwa with putrefying tissue, could also be effected by fresh liver, even when cooked, indicating in the latter case a non-fermentative reductive mechanism. Lintzel (32) discusses the assumption that a bacterial decomposition of some of the trimethylammonium bases occurs in higher organisms; and concludes, on the basis of extensive feeding experiments with betaine, choline, lecithin, γ-butyrobetaine, carnitine and meat extract, that trimethylamine and its oxide, insofar as they are not ingested preformed, must owe their presence in normal human urine to the activity of intestinal bacteria upon certain of these bases, liberating trimethylamine, which would then appear in the urine for the most part as the oxide (see below).

In feeding experiments on human beings, Lintzel obtained the surprising result that ingested trimethylamine appears almost quantitatively (within 10 per cent) as trimethylamine oxide in the urine, and concludes that we must designate the oxide as the normal end-product

of trimethylamine metabolism in man.[6, 7] On feeding preformed trimethylamine oxide (both as the pure compound and as marine teleostean muscle), he found the majority of it excreted unchanged in the urine, with a moderate rise in trimethylamine.[8] He commented upon the rough proportionality between the oxide and the free amine in the human as indicating some sort of physiological relationship, perhaps with respect to oxidative-reductive processes in the organism; and cited in support of this idea the *in vitro* experiments of Ackermann, Poller and Linneweh (3), who stressed the biological importance of the oxide as a hydrogen-acceptor, particularly with respect to the sulfhydryl group of reduced glutathione.

It is a pleasure to acknowledge the capable assistance of Gordon Spence, who accepted full responsibility for the laborious task of catching, handling and collection of urine from the sculpins and flounders used for Specimens 1 and 2.

Summary

It has been shown that approximately half of the total nitrogen in the urine of the sculpin (*Myoxocephalus octodecimspinosus*) and the flounder (*Pseudopleuronectes americanus*) cannot be accounted for by the ordinary nitrogenous constituents. Trimethylamine oxide is present, and accounts for a large portion of this unknown fraction in the sculpin, but is either absent or present in only very small quantities in the urine of the flounder. Brief supplemental studies demonstrated the presence of trimethylamine oxide, in considerable amount, in the urine of the daddy sculpin (*M. scorpius*) and confirmed its presence in the urine of the goosefish (*Lophius piscatorius*). In the discussion the literature concerning the occurrence and significance of trimethylamine oxide and trimethylamine is reviewed.

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[6] Interesting in this regard is the finding (24) that when trimethylamine was injected into carp (in which neither the amine nor its oxide normally occurs) it could be recovered in part from the urine, but that no trimethylamine oxide was formed.

[7] Langley (31) had previously claimed that rabbits metabolized the large majority of ingested trimethylamine, the remainder being excreted unchanged in the urine. He further concluded that part, if not all, of the metabolized amine seemed to be excreted as urea.

[8] Suwa (44) obtained essentially similar results on feeding experiments with rabbits. When the oxide was injected subcutaneously, on the other hand, it was excreted in part as trimethyl- and dimethylamine, but he could detect no unchanged oxide in the urine.

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THE EFFECT OF X-RAYS ON CLEAVAGE IN ARBACIA EGGS: EVIDENCE OF NUCLEAR CONTROL OF DIVISION RATE

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There has been much speculation as to what part of the cell, after exposure to short wave-length radiation, is most responsible for bringing about certain observable biological changes—changes in rate of growth, rate of mitosis, and the like. Also, there has been much speculation as to the influence of the nucleus, if any, on the rate of cell division. The present investigation gives information on these seemingly unrelated questions.

In previous reports (Henshaw, 1932, 1933) we have described the effects of X-rays on the time of the first cleavage in the eggs of *Arbacia punctulata* when irradiated before fertilization. At this time we wish to supplement this by describing the effects when sperm alone are irradiated, and also, when both eggs and sperm are irradiated. As a background for the present work the conditions and findings of the previous work will be reviewed briefly.

Arbacia eggs can be collected in large numbers from a single female; likewise, sperm can be collected in large quantities from a single male. If arranged properly in sea water, the eggs may be fertilized with the sperm and normal development observed. If a few drops of sperm suspension are added to a sample of eggs and the time for cleavage noted, the first cleavage will be found to have occurred in half of the eggs in about 55 minutes under ordinary conditions. If these conditions are kept the same for various samples of a given collection, the cleavage time (i.e., the time from the moment of insemination to the moment when 50 per cent have cleaved) will be constant within one minute ordinarily.

If, however, the eggs are exposed to X-rays immediately before fertilization, the onset of the first cleavage is noticeably delayed. Thus, the radiation in some way affects the protoplasm of the eggs and as a result the first cellular division is slowed. If graded doses of radiation are given to a series of samples, the amount of delay will be found to increase as the dose of radiation increases. In the present study the same irradiation effect (cleavage delay) has been produced

when sperm alone were irradiated. Because sperm and eggs differ distinctly in certain respects, it has been possible to obtain some indication of what part of the cell is affected by the radiation in bringing about cleavage delay, and also something of the relation of cell parts to cell function.

Procedure and Results

Arbacia were collected during the spawning season and maintained in running sea water until needed for use. When material was required, the ovaries were removed from a single female and allowed to shed eggs into a large volume of sea water (several hundred ml.). The ovarian tissue was removed by filtering the suspension through cheesecloth and the eggs were then allowed to settle. Three drops of heavy egg suspension were placed in each of several small glass vessels and the material was then ready for treatment. The vessels had flat bottoms and were about 8 mm. in diameter. The egg suspension was about 2 to 3 mm. deep in the vessels.

Testes were removed from a single male and minced slightly with fine pointed forceps. Portions of the resulting material were then placed in other glass vessels of the same design to a depth of 2 to 3 mm. and were thus ready for treatment.

By laying out the work properly, it was possible to obtain data for three types of experiments at the same time on different portions of the same collection of material—data for (1) irradiated eggs and normal sperm; (2) irradiated eggs and irradiated sperm; and (3) normal eggs and irradiated sperm. It was necessary, therefore, to have on hand both irradiated and non-irradiated material. Accordingly, some of the vessels of sperm and eggs were not treated. The samples receiving treatment were arranged in a special way for exposure to the radiation. Since the time factor has been shown previously to have an influence on the results obtained, the same duration of exposure (30 minutes) was given to all samples. Varying doses of radiation were obtained by placing the samples at varying distances from the source of radiation.

One sample each of sperm and eggs was placed at the distances of 20, 28.3, 40 and 56.5 cm. from the center of the target of the X-ray tube. These distances were selected in accordance with the Inverse Square Law, reducing the intensity by one half in each case. Since means were not available for measuring the intensity at each distance, the relative dosages must be regarded as only approximate. This, however, does not interfere with the relative effects obtained on sperm and eggs, which is the main point of interest here. The intensity at

20 cm. distance was approximately 720 rœntgens per minute, knowledge of the exact amount not being essential. The radiation was supplied by a standard, Coolidge Type, air-cooled, tungsten target, X-ray tube. The conditions under which the radiation was obtained were 120 kv., 180 pv., 5 ma. and a filter of thin paper.

After exposure the eggs were arranged in Syracuse dishes in a larger volume of water and sperm suspension in proper dilution was

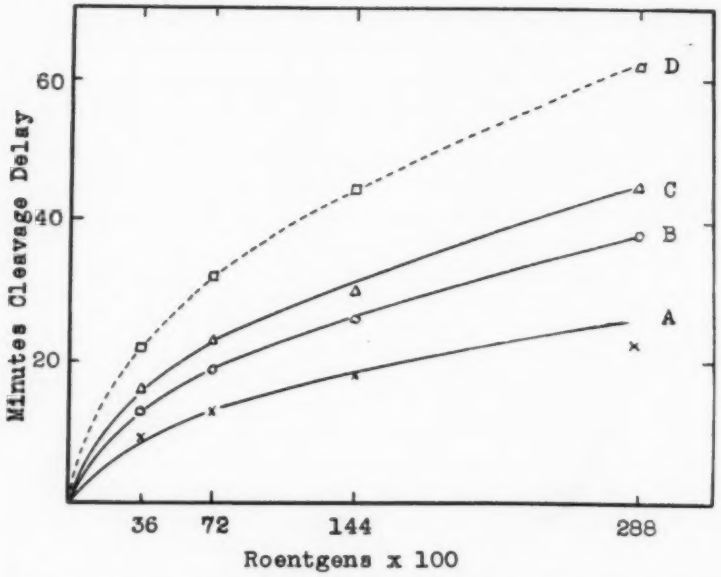

FIG. 1. Curves showing how irradiation effect (cleavage delay) varied with respect to dose of radiation administered. Curves *A* and *B* show the results obtained when eggs and sperm, respectively, were irradiated, while Curve *C* shows it when both eggs and sperm were irradiated. Curve *D* shows the effect which would have been obtained if the effects produced in sperm and eggs had been entirely additive.

added to make the combinations mentioned. Insemination of the different samples was accomplished as nearly simultaneously as possible, never more than two minutes being required for this. Determination of the cleavage time was then carried out according to the procedure described previously.

A series of nine experiments have been carried out, all of which show essentially the same thing. Averaged results for three of these, carried out under precisely the same conditions, are shown in Fig. 1.

The abscissæ indicate dose in rœntgens, while the ordinates indicate irradiation effect in minutes cleavage delay. Curve *A* shows the effects obtained when eggs alone were given increasing amounts of radiation. This, in general, is like the curves obtained previously for similar conditions. Curve *B* shows the effect when sperm alone were irradiated and Curve *C* shows it when both sperm and eggs were irradiated.

It will be seen, not only that cleavage delay is produced by the irradiation of sperm alone, but also that the amount of delay is greater for a given dose when sperm are irradiated than when eggs are irradiated. While the actual amount of difference varied somewhat from experiment to experiment, the ratio of difference obtained for the two gametes remained approximately the same. Also, it will be seen that still greater effects are obtained when both eggs and sperm are irradiated. This indicates that the effects are to some extent additive when both elements are treated.

Discussion

The significance of this investigation may best be considered by analyzing step by step the processes involved. Experiments with irradiated eggs will be taken up first.

An affecting agent, radiation, was administered to a certain type of cell, the *Arbacia* egg, and changes of some kind were produced. These were made evident, at least in part, by the addition of sperm and the determination of the time required for cleavage. The sperm in such a case does two things of consequence. First, it enters the egg and incites it to division, thus initiating the reaction by which the irradiation effect is detected and measured. Second, it carries into the egg a portion of protoplasm which has not been irradiated. Hence, since radiation was allowed to affect only the egg, the procedure of adding sperm and timing cleavage may be regarded mainly as a means used in detecting irradiation effect. Changes produced in the egg, therefore, must have been responsible for the slowing of division observed.

When the same effect is obtained by irradiating sperm, quite a different problem is presented for consideration. A protoplasmic part which had previously been used to detect irradiation effect was caused to harbor changes brought about by radiation, carry them into a portion of non-irradiated protoplasm and there exert an influence which caused the untreated protoplasm to act as though it had been treated. This unique finding may be used in analyzing the action of radiation on the rate of cell division.

In the work with irradiated eggs, no information was obtained which indicated what parts of the eggs were affected by the radiation which, in turn, were responsible for the slowing of division rate. Modifications may have been produced in the membranes affecting permeability; they may have been produced in the cytoplasm causing alterations in viscosity, pH and other chemical organization; or they may have been produced in the nucleus, bringing about structural and chemical modifications. The situation, however, is different when sperm alone are irradiated.

When the normal egg is fertilized with normal sperm, the sperm penetrates the egg membranes, leaving its swimming organ at the surface. Thus, only the sperm nucleus and a small amount of cytoplasm actually enter the egg. The sperm nucleus moves forward through the egg cytoplasm to meet the egg nucleus and the two, so far as is known, share equally in the subsequent nuclear activity of the organism. In regard to the sperm cytoplasm which enters the egg, no important function has been attributed to it except one. It furnishes the functional centrosome for the first division figure, that of the egg not taking part. On entering the egg, the centrosome divides and moves to the poles of the egg, giving rise to the astral arrangement as it does so.

Thus certain points become clear. The irradiation effect which was transported to the egg by the sperm must have been carried by either the nucleus or centrosome or by both. From the fact that cleavage delay can be produced by irradiating sperm alone, it is clear that the delay does not result from alterations produced in the membranes nor in the greater bulk of cytoplasm such as that contained in the egg. Moreover, by considering the fact that the same irradiation effect is obtained when either sperm or eggs are irradiated, and the fact that the egg centrosome is not functional, the possibility of the centrosome harboring an irradiation effect which influences division rate is greatly diminished. The above results, therefore, may be cited as evidence indicating that the irradiation effect, as manifested by cleavage delay, is due to changes produced in the nucleus.

If this be true, the same evidence demonstrates in a clear manner that the nucleus exerts at least some control over the rate of cell division. X-rays have been used previously as a unique surgical tool in performing successful and effective operations on the nucleus, thereby bringing about changes in hereditary activity (Muller, 1928; 1933). The above experiments indicate that physiological activity may also be effectively modified by influencing the nucleus with this agent.

From a purely biological point of view, it is interesting to note that irradiation modification may be produced in a cell containing only half the normal number of chromosomes and that the effect becomes expressed in a cell containing the full number. Here, both an injured and a normal nucleus (haploid) are present in the same cell. It might be expected in such a case that the normal nucleus would exert a dominance over the abnormal one and thus prevent the irradiation injury from being expressed. This, on the contrary, is an example of an abnormal nucleus showing dominance over a normal one.

As yet nothing has been said in regard to the relative susceptibility of sperm and eggs to the radiation. The results, as presented, indicate that the effect is definitely greater when sperm are irradiated. If, as believed, the egg and sperm nuclei share equally in the nuclear functions of the organism, and if it is true that nuclear changes brought about by radiation are responsible for the radiation effect observed, it would be expected that equal effects would be produced on sperm and eggs by equal doses of radiation. The differences observed, however, are appreciable and tend to indicate a differential response on the part of the gametes. But there are reasons for believing that the differences observed are not real.

First, Heilbrunn and Young (1935) in a recent paper have reported work done on the same material and with the same source of radiation. In brief, they irradiated eggs in sea water in one case and in ovarian tissue in another and found noticably more cleavage delay when ovarian tissue was present during treatment. They suggest that this is evidence indicating the presence of *necrohormones*—an irradiation product which is produced in one tissue and which is diffused out and influences another. As a matter of convenience in our experiments, the eggs were irradiated in sea water and the sperm were irradiated in the presence of testicular material. Should necrohormones have been produced by this material, they would have caused the irradiation effect to be greater than it otherwise would have been. Second, the discrepency may be accounted for by differences in absorption in the two cases. The eggs settled to the bottom of the vessel during treatment and were protected by two to three millimeters of sea water, while at least part of the sperm were held near to the exposed surface by the testicular material. Since unfiltered radiation was used, two millimeters of sea water probably removed an appreciable amount of otherwise effective radiation. These factors, however, have not been investigated, thus making it impossible to discuss their significance at the present time.

Perhaps the most interesting phase of this investigation lies in the

set of facts implied by the quantitative results. The reaction in its simplest form appears to be one of indirect dependence. That is, the effect observed (cleavage delay) is dependent on the amount of radiochemical change of a particular kind which is produced in the living system and which in turn is dependent on the amount of radiation administered. How the observed effect varies with respect to the quantity of radiation given is indicated by the shape of the curves obtained, but how the radiochemical change varies with respect to the dose of radiation applied and how the observed effect varies with respect to the amount of radiochemical change, is less obvious. The data, however, may be analyzed to obtain certain indicative information on these points.

Since it has been possible to produce the same irradiation effect by exposing either gamete, it is probable that the same radiochemical change (that which causes cleavage delay) is produced in the sperm and eggs indifferently. Through fertilization, then, two quantities of radiochemical change, known to produce definite amounts of cleavage delay, are brought together in the same system where their combined effects are expressed as one. This unique procedure, made possible only by the fact that cleavage delay may be produced by irradiating either gamete, offers an indirect method of investigating the quantitative aspects of the radiochemical change involved. For example, if the combined effect observed is equal to the sum of the two effects obtained independently, it follows that the observed effect is directly proportional to the amount of radiochemical change whatever it may be. Curve *D* in Fig. 1 shows the combined effects obtained by adding Curve *A* to Curve *B*.

The curve actually obtained when both elements were irradiated falls considerably below Curve *D*, indicating that the relation is not directly proportional. However, Curves *C* and *D* appear to be similar in shape, which indicates that the observed effect is equal to the combined calculated effect times some constant less than one. This has been found to be very nearly 0.72 at various points along the curves in the figure. Insofar as these results are trustworthy, this indicates that a linear relation exists between the radiochemical change produced and the biological effect observed. Since, however, the quantitative data now available are limited, it seems best not to carry the analysis farther at this time nor to emphasize the relationships observed. At this time it is sufficient to point out that a new method is available for investigating certain phases of the radiobiological reactions about which very little is known.

Summary

1. Experiments have been carried out to investigate the influence of X-rays on the division rate of *Arbacia* eggs.

2. It was found, first, that the time required for the first cleavage was noticably prolonged when the eggs were irradiated before fertilization.

3. Second, it was found that the same effect could be produced by irradiating the sperm before they were allowed to fertilize normal eggs. Thus, an irradiation effect was carried by one gamete into another where the effect became expressed. The non-irradiated gamete was, therefore, caused to behave as though it had been irradiated.

4. Irradiation of both eggs and sperm indicated that the effect obtained was to some extent additive.

5. It was pointed out that only the nucleus and centrosome of the sperm appear to have an important influence on the egg and that the irradiation effect in such a case must have been carried by one or both of these. Reasons were given for believing that the centrosome was not important in this respect, thus indicating that the slowing of cell division observed was due to irradiation effects produced in the nucleus.

6. It appears, therefore, that the nuclei of the cells studied exert at least some control over the rate of cell division, and it is significant that such control is manifested when the damage is produced in a haploid nucleus and the effect is expressed in the presence of a normal haploid nucleus.

7. A method is presented for investigating quantitatively some of the more remote aspects of the radiobiological reactions.

Acknowledgments: The authors wish to express their indebtedness to Dr. G. Failla for his interest and coöperation throughout the course of this work.

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RECIPROCAL CHORIO-ALLANTOIC TRANSPLANTS OF EMBRYONIC DUCK AND CHICK KIDNEY TISSUE

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The results of reciprocal heteroplastic transplantations are not always the same. Such observations have appeared sporadically in the literature, particularly pertaining to grafting experiments between different species of amphibians. Similar observations on warm-blooded vertebrates are not as common. Loeb (1920) grafted kidney tissue between various forms. Some of these grafts were made reciprocally, insofar as host-donor relationships were concerned, between pigeon and guinea pig, and rabbit and guinea pig. More recently, Loeb and King (1935), and Loeb (1935*a* and 1935*b*) made a number of reciprocal transplantations which included such combinations as Gray Norway rats and mutant races, rat and mouse, rat and guinea pig, cat and rat, and rat and pigeon. Adult forms were used in these experiments, which involved transplants of thyroid, cartilage, ovary, and closely associated tissues. In general, the results showed that the grafts between the more closely related forms were more favorable than those between the distantly related forms. In the former there was some indication that a parallelism existed between the intensity of the reaction and the genetic relationship of the host and donor. In the latter, it was difficult to demonstrate this parallelism because necrosis appeared so rapidly in the transplanted tissue that it interfered with the more significant reactions which appear against living tissue. Under these conditions the results of the reciprocal transplants were, for the most part, similar, but some differences were noted.

Sandstrom (1932, 1934, and 1935), although not concerned with reciprocal transplants, reported a series of experiments designed to study the problem of heteroplastic grafting. His experiments were limited to that period in embryonic development when both the host and donor developed those properties responsible for the incompatibility displayed in adult tissue grafts. His results demonstrated that, in addition to the species specificity of the tissues, a number of interrelated factors must be considered in interpreting the results, e.g., the degree of differentiation of the donor tissue, the size of the transplant, the rate of incorporation of the transplant by the host, the capacity of

the transplant to function in its new environment, and the possibility that the grafted tissue might become adapted to the host tissues.

The previous experiments (1932, 1934, and 1935) having been concerned with duck kidney tissue transplants to the chorio-allantoic membrane of the chick, the question arose as to whether grafts of the reverse host-donor relationship (i.e., metanephric tissue of the chick embryo grown on the chorio-allantoic membrane of the duck) would give similar results, and if not, whether a comparison of the reciprocal grafts would give any additional information pertaining to the general problem of heteroplastic transplantations.

A preliminary report on experiments, made with this thought in mind, has already been presented in abstract form (Sandstrom, 1933). During the interim additional transplants were made to fill gaps in the experimental series, and to corroborate earlier observations.

Material and Methods

The procedure used in these experiments was similar to that described by Sandstrom (1934). Briefly, it consisted of implanting a small bit of donor tissue on the chorio-allantoic membrane of the host where it was allowed to grow for the desired length of time, within the limits prescribed by this method of experimentation. In the present set of experiments, however, reciprocal transplants were made between duck and chick embryos, i.e., duck tissue was transplanted to the chick membrane, and chick tissue was transplanted to the duck membrane. The metanephros was again used as a source for donor material because it is an actively functional tissue (in contrast to cartilage, for example, which is considered passively functional), and, under proper incubation conditions, is known to differentiate in either homoplastic or heteroplastic chorio-allantoic grafts (Sandstrom, 1932). The donors were White Pekin duck embryos of 13, 16, and 24 days incubation, and single-comb White Leghorn chick embryos of 9, 13, and 19 days incubation. The hosts were White Pekin duck embryos and single-comb White Leghorn chick embryos of 14 and 9 days incubation, respectively.

Since the degree of differentiation is an important factor in determining the capacity of tissue to grow on the chorio-allantoic membrane, it was necessary, in order to make valid comparisons between the reciprocal transplants, to select hosts and donors of approximately the same stage in development. This is, of course, a problem in itself. The question immediately arose as to whether the chick, which hatches on the twenty-first day of incubation, is in the same stage of development at the time of hatching as is the duckling which hatches on the

twenty-eighth day of incubation. Or is the latter more developed because of the additional week in the shell, and therefore equivalent to the one-week old chick? Either a gross, or histological study of the embryonic structures, such as is used in determining the degree of development in the earlier somite stages of avian embryology, for example, is of little value after visible differentiation has ceased. Since the metanephros was used again as donor tissue, and for the want of a better method in determining equivalent development, it was assumed that the changes occurring in the physico-chemical nature of the epithelial cells of the proximal convoluted tubules, as indicated by the manner in which they stored vital stains, would approximate the desired criterion. Hurd (1928) showed that trypan blue was stored in the form of bright granules in the metanephros of the chick embryo of 19 days incubation. Using the same kind of dye, Sandstrom (1935) determined that it was stored as well-defined masses in the kidney of the duck embryo of 24 days incubation. Assuming that these stages, therefore, are of equivalent development, from the ratio thus established, it was inferred that the chick and duck were approximately equivalent at the time of hatching. On this basis, insofar as it was practical, the ages of the hosts and donors used in the reciprocal transplantations of these experiments were selected.

The grafts were removed at 24-hour intervals after an initial period of 48 hours. They were fixed in Zenker's formal, sectioned at 6 and 8 micra, and stained with methylene blue-eosin. The present report is based on the study of 233 grafts.

Comparison of Grafts

Series I—Chick and Duck Donors of 9 and 13 Days Incubation, Respectively

At the time of transplantation, the metanephric tissue of the donors, whether chick or duck, possessed scattered tubules, and sometimes occasional renal corpuscles. It was still able to continue its differentiation when implanted on the chorio-allantoic membrane of the host, for considerable metanephrogenous tissue was still present. The actual condition of the transplanted tissue, however, varied according to the length of time the grafts were allowed to grow.

A comparison of the grafts of metanephric tissue of the chick embryo grown on the chorio-allantoic membrane of the duck for 2, 3, and 4 days, with those of the duck grown on the chick membrane for identical periods, showed similar conditions. All of the grafts were well incorporated. Differentiation had not progressed to any great extent in those removed on the second day; it appeared much as it did

at the time of transplantation. In the grafts removed on the third and fourth days, the amount of metanephrogenous tissue was considerably reduced, and the transplants consisted largely of uriniferous tubules which, in some instances, could be differentiated into their various parts. But the tissue in the grafts removed up to this time was not all normal, particularly in those taken on the second day. Centrally, it appeared as though in the initial stages of necrosis. Instead of taking the methylene blue stain as did the more peripheral normal tissue, it stained with eosin only. The cytoplasm of the cells of the tubules contained vacuoles, and the nuclei were shrunken. This condition was undoubtedly due to the inability of the blood vessels to penetrate into the transplant rapidly enough to insure proper vascularization. Accompanying this apparently necrotic condition were numerous small lymphocytes. By the third and fourth days there was considerably more normal, and less necrotic, tissue. At the same time there occurred a proportional reduction in the number of small lymphocytes. They were usually limited to the small areas of tissue in which necrosis had definitely set in.

Upon comparing the reciprocal grafts removed on the fifth and sixth days, one prominent difference was noted which was not apparent in any of the transplants removed earlier. The incorporation of the metanephric tissue of the chick into the chorio-allantoic membrane of the duck was less complete than that of the duck into the chorio-allantoic membrane of the chick. The failure of the chick tissue to become completely incorporated manifested itself in two ways. In some instances, the grafts were taken into the host membrane, but only a small part of the surface of the transplant came in contact with the host tissue, and the region where the loose connective tissue and blood vessels of the chorio-allantoic membrane could enter was therefore limited considerably. Usually this contact was confined to one side. The remaining surface of the graft was separated from the host tissue by a space which was occasionally filled with a colloid-like substance. The tissue was normal.

In other instances, the grafted tissue was not completely incorporated. The impression gained from an examination of the sectioned material was that the chick tissue, although at first fully incorporated into the duck membrane, was being forcibly ejected. The inner side of the transplant was in contact with the host tissue, and the loose connective tissue and blood vessels of the host membrane entered the graft in this region only. The inner part, therefore, was, in a sense, incorporated, and was normal in all respects. But in passing toward the outer part, the tissue became more and more necrotic. The

tubules were crowded, and their shapes altered considerably. It was still possible, nevertheless, to detect that some differentiation had taken place on the membrane. The outer part of the graft was definitely necrotic. It was not covered by the chorionic epithelium, the loose edges of which were found about the periphery, and the dead tissue was sloughing into the space between the chorionic surface and the inner shell membrane.

It is to be understood, however, that incomplete incorporation, such as described above, was not universal among the grafts of chick tissue on the duck membrane. Other grafts, in the minority to be sure, were incorporated fully as well as the duck-on-chick transplants. Regardless of the host-donor relationship, those grafts that were sufficiently incorporated to insure proper vascularization to all regions showed a high degree of differentiation with all parts of the typical kidney present, and little necrosis. Lymphocytes were not common, even in the grafts containing necrotic tissue.

There were more notable differences between the reciprocal grafts that were removed from the seventh to the tenth days. Although most of the tissue in the duck transplants grown on the chick membrane was normal, conditions were quite variable in the grafts of the reverse host-donor relationship. A few small grafts contained normal chick tissue, others showed varying amounts of necrotic tissue, and still others were in the process of sloughing in the manner already described. In some cases the transplanted chick tissue had completely sloughed from the host membrane, and the only evidence of a previous graft was an increased amount of connective tissue in the general region. In addition, there was another striking difference between the two types of transplants. In the majority of grafts of chick tissue removed from the duck membrane during this period, intense infiltrations of granulocytic leucocytes and many small lymphocytes made their appearance. Generally granulocytes are not uncommon in metanephric tissue grafts, but they are usually present in small numbers only. They are normally formed from the interstitial connective tissue of the developing metanephros, and their existence in the transplant is to be expected. But in the chick grafts removed from the duck membrane on, or after, the seventh day, they manifested themselves in a manner not found in any of the duck-on-chick grafts. They were so numerous that the transplant, in some instances, was completely obscured. They were also found in the connective tissue of the chorioallantoic membrane immediately surrounding the graft. Wherever the granulocytes were sufficiently scattered so that the transplant could be studied adequately, its tissue was found completely incor-

porated and, for the most part, histologically normal. Some necrotic tissue, however, was present. The intensity of the granulocytic infiltrations could not be correlated with the amount of necrosis, for grafts which appeared almost completely normal were as intensely infiltrated as those that contained large necrotic areas.

A study of the chorio-allantois immediately around the grafted tissue revealed the source of the great numbers of granulocytes. It was quite obvious that the presence of the foreign tissue had stimulated the formation of granulopoietic centers, much in the same manner that homoplastic chorio-allantoic grafts of adult chicken spleen stimulated the formation of centers of granulopoiesis as described by Danchakoff (1918). In fact, many of the stages in the differentiation of the granulocytes, as described by her, were found. The loose connective-tissue cells had enlarged to form relatively large amœboid cells—the hemoblasts. Various stages could be selected to form a sequence of transformations leading from the hemoblast to the granuloblast. The final stage in this selected line of differentiation, the granulocyte, was very common. All of these stages were limited, for the most part, to circumscribed areas. These loci were located at various distances from the grafted tissue. In some cases they were directly adjacent to the transplant so that the various stages in the differentiation of the granulocyte were within the grafted tissue. More often, the loci, while in the immediate vicinity, were slightly removed from the graft, and in these cases, only fully differentiated granulocytes were found in the transplant.

Small lymphocytes, as well as granulocytes, were present, both in the grafted tissue and surrounding host membrane. They were not as common as the granulocytic leucocytes, and were somewhat larger than the small lymphocytes found in the necrotic-appearing areas of grafts removed 2 days after implantation. No evidence was found which would indicate the loci of their formation as coincident with the granulopoietic centers, although small lymphocytes were present in large numbers in these centers. Unlike the results of Danchakoff (*loc. cit.*), no convincing sequence of cell types could be selected that would suggest their transformation from the hemoblasts.

Series II—Chick and Duck Donors of 13 and 16 Days Incubation, Respectively

The reciprocal transplants of this series showed decided differences, although at the time of transplantation, the metanephroi of both donors had reached a comparable degree of histological differentiation. The metanephric tissue of the duck continued its growth and development on the chick membrane in the manner reported on previous occasions

(Sandstrom, 1932 and 1934). Considered briefly, the condition of the transplant varied according to the length of time the graft was allowed to remain on the host membrane. There was an initial period of 2 or 3 days when the final stages of incorporation were in progress, after which the implant was completely taken into the chorio-allantoic membrane and covered by the chorionic epithelium. During this period, the peripheral tissue was apparently normal, whereas the central part showed the early stages of necrosis, both as to its structural appearance and staining reactions. Numerous small lymphocytes were present in, and about, the necrotic area. Grafts removed subsequently, e.g., from the fourth to the tenth days, showed progressively less necrotic, and more normal tissue, as the length of time they grew on the membrane increased, so that ultimately their tissue, for the most part, was comparable to that of a normally developed metanephros of equivalent age. But some necrosis was present even in the grafts removed during the later intervals, the extent varying from small areas to complete grafts. The number of grafts containing necrotic tissue, however, was decidedly in the minority.

Upon comparison, the grafts of the reverse host-donor relationship, i.e., metanephric tissue of the chick grown on the chorio-allantoic membrane of the duck, showed conditions quite in contrast to the duck-on-chick grafts. A most striking difference was apparent in the process of incorporation, which, in most cases, was considerably retarded. Regardless of the length of time the grafts were allowed to remain on the duck membrane, the implanted tissue was rarely completely incorporated. It remained, more or less, in a stage comparable to that found in the duck-on-chick transplants removed from the host membrane within 48 hours after implantation. The epithelial cells of the membrane, undoubtedly stimulated in some manner by the presence of the implanted tissue, increased in number, forming epithelial projections which extended into the implant. Thus, that part of the metanephric tissue which was in immediate contact with the chorionic epithelium became incorporated first; it was "engulfed" by the host epithelium. Peripherally, the chorionic epithelium folded upward about the transplant, much as the somatopleure folds over the developing chick embryo in the formation of the amnion and chorion. Usually these folds extended upward only a short distance. Rarely did they cover the graft completely, and if so, not before the eighth day. In these cases, the inner part of the fold was sometimes found, much folded, in the mesoderm of the membrane, and not infrequently formed a hard, cornified mass.

Obviously, the tissue of the transplant was considerably affected

by the retarded incorporation. That part which was "engulfed" by the epithelium was normal in all respects. It procured sufficient vascularization with little delay, and continued its development, so that the grafts removed from the eighth to the tenth days, for example, contained apparently normal, fully differentiated metanephric tissue. Necrosis appeared rapidly in the remaining unvascularized part, which was usually left outside the membrane, although occasionally it was completely covered by the chorionic epithelium. When covered, the dead tissue was always separated from the normal either by a space, or connective tissue.

Series III—Chick and Duck Donors of 19 and 24 Days Incubation, Respectively

The high degree of differentiation that the donor tissue, whether chick or duck, had attained at this stage of development, was reflected in the incapacity of the transplants to grow on the chorio-allantoic membrane. Regardless of the host-donor relationship, no great amount of normal tissue was found in the grafts. That which was present probably came from small scattered areas of metanephrogenous tissue that were still present at the time of transplantation, or represented tissue which was sometimes found to persist through the period that the grafts remain on the host membrane. Only one graft, a chick-on-duck, was completely normal.

One prominent difference was apparent between the reciprocal grafts of this series. The metanephric tissue of the chick was almost always completely incorporated into the duck membrane, even though it was seldom capable of continuing its growth. This complete incorporation of the implant was in striking contrast to the conditions found in similar grafts of the previous series (Series II), where incomplete incorporation was almost universal regardless of the stage of removal. It was also in striking contrast to the results obtained in the reciprocal grafts, i.e., duck-on-chick, of this series, which resembled, to a degree, the conditions prevalent in some of the chick-on-duck grafts of Series I. During the early period of incorporation, the implanted tissue, completely taken into the chick membrane, consisted of the characteristic peripheral area of normal tissue and the relatively larger central area of apparently necrotic tissue. Numerous small lymphocytes were aggregated in and about the central part. From the fourth day on, however, much of the grafted tissue was found in the process of sloughing. Not infrequently, it had sloughed from the membrane completely, and the area once occupied by the duck tissue was filled with connective tissue.

Discussion

The foregoing comparison of the embryonic duck and chick transplants demonstrated that a definite reciprocal relation existed between the host and donor species only during early embryonic development. During this period the metanephrogenous tissue of both the duck and chick were equally well incorporated into the chorio-allantoic membrane of their respective hosts. As development proceeded, however, this reciprocal relation was, to a considerable extent, lost. Pronounced differences appeared in the transplants. For example, the implanted metanephric tissue of the 14-day chick embryo was incorporated less completely, and more slowly, than the equivalently developed tissue of the 19-day duck embryo was into the chick membrane. This retarded incorporation is demonstrated, not only in many of the grafts of Series I (donors of 9 days incubation) that were removed from the host membrane on, and after, the fifth day, but somewhat more decisively in most of the grafts of Series II (donors of 13 days incubation), irrespective of the time of removal.

Other differences were noted. In Series I, completely incorporated chick tissue, after having grown in the new environment from 7 to 10 days, often stimulated the formation of leucopoietic centers in the neighboring regions of the duck membrane. No stimulation of this kind resulted from the transplantation of duck tissue to the chick. Furthermore, in Series III, the implants of fully differentiated, functional metanephric tissue from both the chick and duck (ages of 19 and 24 days incubation, respectively) were completely incorporated, with no obvious delay, into the membranes of their respective hosts. In this group, however, it was the transplanted chick tissue that grew, or at least persisted, in its new environment, whereas the grafted duck tissue, after having grown for a few days, frequently sloughed from the membrane without creating any apparent disturbance in the neighboring host tissue. And finally, closely correlated with the retarded rate of incorporation, considerably more necrotic tissue was found, in general, in the grafts of chick tissue than in those of duck tissue.

Undoubtedly, the reactions described in the foregoing paragraph were associated with the development of species specificity in the donor tissues. Its appearance in the metanephric tissue of the chick obviously occurred on the fourteenth day of incubation, the time when the incorporation of the chick tissue was first retarded. But as for the appearance of species specificity in the duck tissue, no actual evidence is available from these experiments. Assuming that the method used in determining equivalent development is essentially

correct, it should be present at approximately the nineteenth day of incubation. As a matter of fact, this conclusion checks with that of Sandstrom (1934) who, from a study of heteroplastic and homoplastic chorio-allantoic grafts of duck kidney tissue, experimentally demonstrated its presence at this time.

Although it is essential that the transplanted metanephric tissue should function in order to continue its growth on the chorio-allantoic membrane, as was shown by Sandstrom (1934 and 1935), apparently its incapacity to do so does not elicit a reaction from the host. At least this was true for the duck-on-chick transplants. More than likely, therefore, the lack of function in the chick-on-duck transplants cannot be considered an important factor in causing the reactions found in these grafts. That the metanephros of the chick has developed function by the fourteenth day of incubation is suggested by the results of Gersh (personal communication). By a refined technique, he has been able to demonstrate activity in the tubules of the chick metanephros as soon as they are structurally differentiated, i.e., starting the eleventh day. According to his observations, however, only a very few tubules are sufficiently differentiated at this time, and the number increases but slowly, making it highly improbable that the incapacity to function played an important part in instigating the reaction obtained.

Since the species specificity of the transplanted tissue was the primary factor responsible for the reactions between graft and host, the differences in these reactions, as found upon comparison of the reciprocal grafts, must be ascribable to differences in the capacity of this factor in the donor tissues to instigate reactions in the membranes of their respective hosts. The more determined reactions obtained from the chick implants, as compared with the duck, suggest that either the species specificity factor of the former is the more potent of the two, or the chorio-allantoic membrane of the duck is the more reactive. In either case, it would be the donor that provoked the reaction, the character of which would necessarily have to be a function of the host. The reactions obtained in the reciprocal grafts, therefore, differed. These results are well in accord with the statement made by Loeb (1930, p. 572) that "if the relation between host and donor is reversed, the results of transplantation, as manifested in the intensity of the reaction against the transplant, do not need to be identical; no simple reciprocity therefore exists. This can be readily understood, if we consider that it is the host which reacts against the transplant, and not the transplant which reacts against the host, or at least that, under ordinary circumstances, we have no means of determining the reaction on the part of the transplant against the host."

Incidentally, it is difficult to understand how the donor tissue, without reacting against the host, can provoke a reaction in the host, as implied in the statement by Loeb, quoted in the previous paragraph. As a matter of fact, an analysis of the process of incorporation, as it occurred in the chorio-allantoic grafts of chick tissue on the duck, suggests that the donor does react against the host. Although chorio-allantoic grafting as a method for study of heteroplastic transplantation may possess certain shortcomings, it has the advantage over other methods in that the incorporation of the implant can be used as an index of reactivity. The implant is carefully placed on the surface of the chorionic epithelium, so that the epithelial tissue is involved before the foreign transplant finally becomes lodged in the connective tissue of the membrane. No actual mechanical injury, therefore, is incurred by the host. This method is in contrast to that of subcutaneous transplantation, for example, as practiced with adult animals, in which the implant is placed directly into the subcutaneous connective tissue, thereby mechanically assisting the process of incorporation. In addition, since mechanical injuries to the recipient's tissues are unavoidable results of this procedure, the incorporation of the implanted tissue cannot be used in the study of either host or donor reactions. In the chorio-allantoic grafts under consideration, therefore, the process by which the implant was incorporated assumed considerable importance. The only connection between the host and donor tissues at the time of implantation was a physical contact between the surfaces of the implant and chorionic epithelium, and, it will be recalled from the description of the results, that the epithelium subsequently "engulfed" portions of the implanted tissue. Under these conditions, the rate of incorporation was determined by two opposing factors—an apparently natural tendency for the epithelium to react favorably toward the implant, as indicated by the manner in which it "engulfed" parts of the implanted tissue, and the presence of the species specificity factor in the donor tissue which inhibited the "engulfing" activity of the host. The amount of tissue incorporated was apparently dependent on the relative effectiveness of these two factors. In any event, the analysis has indicated that some of the responsibility for the retarded incorporation rests with the reaction of the transplant against the host.

It has been emphasized that the species specificity factor of the donor tissue was of primary importance in causing the reactions in the transplants; therefore further consideration must be given to the seemingly contradictory results obtained in the grafts of Series III, in which fully differentiated, species specific tissue was not only readily

incorporated, but even failed to call forth a reaction from the host. From the results of the chick-on-duck transplants it was evident that, insofar as its capacity for eliciting a host reaction is concerned, the developing donor tissue passed through three stages: during the first, which obtained previous to the development of species specificity, the host and donor tissues were compatible; the second obtained during the development of species-specific properties which caused a definite reaction; and the third obtained subsequent to the appearance of species specificity when no reaction occurred. Owing to the milder response, these stages, although present, were not very apparent in the duck-on-chick grafts.

Such an unusual sequence is not unparalleled among grafting experiments, for an analogous situation was found existent in some of the results mentioned by Loeb (1930) in a review of his theory of organismal differentials. This author, referring particularly to the intensity of the reaction which appeared against his transplants of non-resistant tissues (e.g., thyroid, made between variously related adults), as indicated by the lymphocytic infiltrations, stated that few, or no, lymphocytes were found in autoplastic transplants. According to Loeb's conception, the lymphocytic reaction is a response toward a toxin produced by the transplant. Inasmuch as host and donor were the same individual, and had identical individuality differentials (chemical characteristics), no toxic substance was produced to attract the lymphocytes. In homoplastic transplants, although the respective individuality differentials of the host and donor differed, this difference was not great enough to interfere with the metabolic activity of the transplant. Sufficient toxin was produced, therefore, to call forth a decided infiltration of lymphocytes. In heteroplastic transplants, the individuality differentials of the host and donor were so different that the body fluids of the former, acting directly on the latter, caused degenerative changes therein. These changes interfered so extensively with the metabolism of the implant that only a slight amount of toxin was formed. As a result, the intensity of the lymphocytic reaction was less pronounced, and appeared more slowly, than that which appeared against the homoplastic transplants.

Insofar as the intensity of the reaction is concerned, therefore, the sequence obtained in the chorio-allantoic grafts of developing metanephric tissue not only seems to recapitulate the conditions described in the preceding paragraph, but the explanation postulated by Loeb, in consideration of his results, may also be applicable. The conditions found in the chick-on-duck grafts of the first stage are comparable to those found in the autoplastic transplants; no reactions occurred. In

the grafts of the second stage, however, the intensity of the reactions resembled that of the lymphocytes in the homoplastic transplants. The individuality differential of the donor had developed sufficiently to excite a reaction without interfering appreciably with its capacity to live in the new environment. Furthermore, the implanted tissue, because it was still undifferentiated enough to be plastic, could be handled, etc., during the operative procedures without causing considerable degenerative changes. Thus this metabolically active tissue, perhaps through the agency of a toxin, affected the host tissues in a decisive manner. In the final stage of the sequence, e.g., grafts of Series III, true heteroplastic conditions were being approximated; the development of the individuality differential (species specificity factor) had reached completion. Even though the differentials of the host and donor resembled one another less than in the previous stage, the host was unresponsive toward the implant during the limited time it remained on the membrane. As in the case of heteroplastic transplants of Loeb, the intensity of the reaction was undoubtedly diminished because of degenerative changes that occurred in the implant. To what extent the body fluids present in the chorio-allantoic membrane of the unhatched host were capable of causing these changes is doubtful. It is more than likely that the changes were caused by injuries incurred during the operative procedure, since the plasticity of the tissue had decreased considerably. In addition, vascularization, so essential to a differentiated, functional tissue, was necessarily retarded because the process of incorporation, as it occurs in chorio-allantoic transplants, at best, is relatively slow. Under such conditions the implant could not have consisted of an actively metabolizing tissue, and thus lacked the essential prerequisite for calling forth a reaction.

Summary

1. Metanephric tissue from duck and chick embryos that were approximately equivalent in development was transplanted to the chorio-allantoic membrane of the chick and duck respectively. The hosts, also, were in comparable stages of development.

2. A reciprocal relation was found to exist during the early embryonic stages only. As development progressed this relation was lost, to a large extent; prominent differences occurred in the two types of transplants. Whereas relatively slight or no reactions were observed in the duck-on-chick grafts, intense reactions appeared in the grafts of the reverse host-donor relationship.

3. The reactions appearing in the grafts were associated with the development of the species specificity factor in the donor tissue.

Its appearance in the chick tissue occurred at about the fourteenth day of incubation.

4. The reactions which appeared as a result of the development of the species specificity factor were: (1) a decided retardation of the rate of incorporation of the implanted tissue; (2) possible expulsion of the transplant from the membrane; and (3) the stimulation of leucopoietic centers in the connective tissue of the surrounding host membrane. These reactions, if present in the duck-on-chick grafts, were scarcely observable because of their mildness.

5. It was demonstrated by these experiments that, although the host reacted against the transplant, which obviously instigated the reaction, it was also possible, as was shown by an analysis of the process of incorporation, for the transplant to react against the host.

6. Seemingly contradictory results were obtained in the grafts of fully differentiated, species specific tissue, in that the implants were readily incorporated into the host membrane, without provoking a reaction. Although no evidence was offered, an explanation of these unusual results was suggested which was based on the theory of Loeb (1930) that the reaction against a transplant is dependent, to a large measure, on the active metabolism of the transplanted tissue.

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EXPERIMENTAL STUDIES ON THE NUPTIAL PADS OF MALE TRITURUS VIRIDESCENS

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During the breeding season the adult male *Triturus viridescens* develops heavy black nuptial pads on the inner aspect of the femoral area and toe tips. There is also a marked hypertrophy of the tail fin, swelling of the cloaca, and development of the hedonic glands (Hilton, 1902). Adams (1932) reported the experimental production of leg-pad development, tail-fin response as well as ovulation and moulting following the administration of anterior pituitary extract (*Phyone*) in this species. Increase in tail-fin size was not interpreted as a secondary sexual character under the influence of the gonad since it can be produced with *Phyone* injections in castrates. These results have been confirmed by Dawson and Jimenez (1933) who, in addition, demonstrated that the hedonic glands responded in the manner characteristic of secondary sexual characters. Similar logic has been applied by Adams (1931) in a study of the effect of intramuscular injections of anuran pars anterior in immature *T. cristatus*. Here dorsoventral expansion of the tail and head enlargement were produced in both normal and castrated animals.

Materials and Methods

The femoral pad is of value in experimental studies as an indicator of the hypophysis-gonad-reproductive tract inter-relationship. The writer was attracted to this area as a useful indicator for studies on the

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Explanation of Figures

1. Ventral view of an animal in which its left femoral pad has been exchanged with the skin of the pectoral area (autoplastic series) showing hypertrophy and pigmentation of normal and transplanted tissue following six injections of pituitary extract. Photograph taken three hundred and thirty-five days after graft was made.
2. Pectoral area of same animal at higher magnification.
3. Ventral view of a newt in which a femoral pad was grafted from another animal of the same species to the pectoral region (homoioplastic series). Typical response of secondary sexual characters was obtained for the normal host tissue following seven doses of pituitary extract. The homoioplastic transplant shows no corrugation or pigmentation. Photograph taken sixty-six days after transplantation.
4. Pectoral area of same animal at higher magnification.

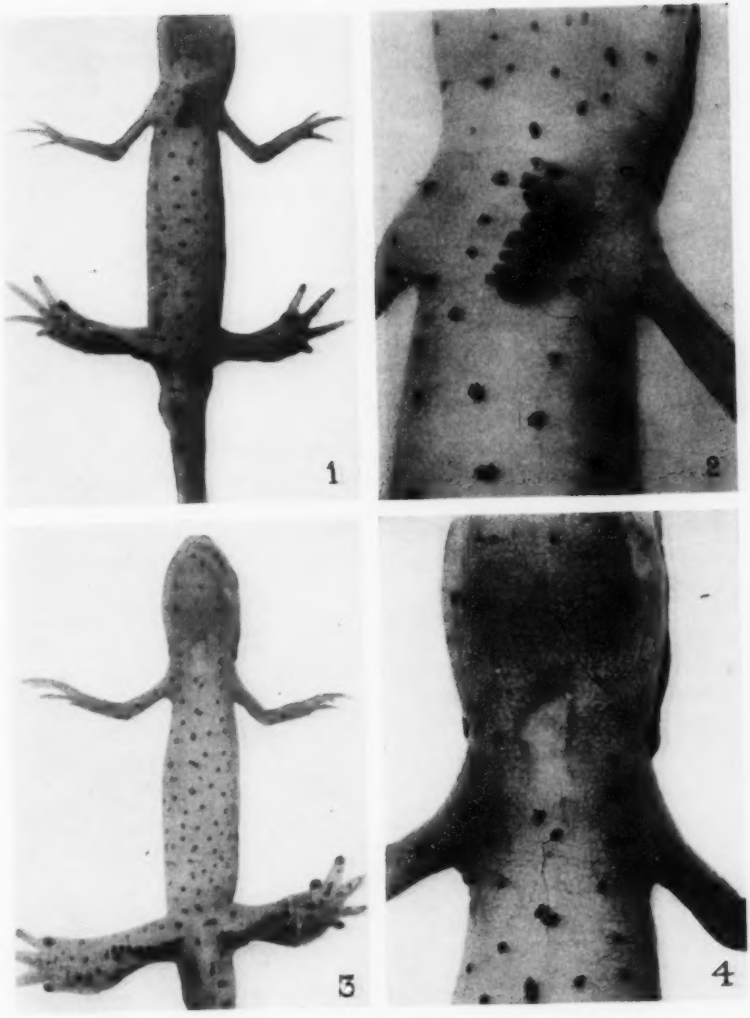

PLATE I

persistence of skin transplants. The general technique employed in this work consisted in cutting out the entire femoral area from one limb of an animal under chloretone anaesthesia and transplanting it to the midventral line between the forelimbs. Upon stimulation with intraperitoneal implants of frog anterior pituitary or an extract of whole sheep gland it was possible to determine whether the grafted tissue had persisted by referring to the pad of the uninjured leg. Only sexually mature aquatic male newts were used. All experimental animals were kept in aquaria at room temperature.

Results

Autoplastic transplants were consistently found to persist unchanged in eight animals after more than three hundred days. Figures 1 and 2 illustrate the results obtained in a typical case three hundred and thirty-five days after autotransplantation. Stimulation of secondary sexual characters was brought about by means of six doses of pituitary extract (whole sheep gland, Parke Davis and Company). Each dose consisted of an intraperitoneal injection of 0.2 cc. of a 1–5 aqueous solution. Injections were made every second day. Adequate response is evidenced (Fig. 1) by the development of hypertrophy and pigmentation of toe pads and the normal (right) femoral area. The left femoral pad is seen in the pectoral position and this appears typical (Fig. 2).

In eight cases of homoiotransplantation the femoral pad was never found to perist unchanged for more than sixty days. Progressive removal or rearrangement of grafted tissue was evidenced by the failure of characteristic hypertrophy of the transplanted pad tissue following pituitary stimulation. Figures 3 and 4 illustrate a typical

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Explanation of Figures

5. Ventral view of an animal one hundred and fifty days after amputation of left limb. An autoplastic transplant of the femoral pad was made at the time of amputation. Graft shows a reaction similar to the normal right leg which serves as control to the action of seven doses of pituitary extract.

6. Ventral view of an animal seventy days after amputation of the right limb. An homoioplastic transplant of femoral pad tissue was made at the time of amputation. The normal left leg shows characteristic reaction to seven doses of pituitary extract but the homoiotransplant seems almost entirely resorbed.

7. View of the ventral aspect of the pelvic area and left limb regeneration seventy days after amputation. The first toe shows definite development of pigmentation in response to seven injections of pituitary extract.

8. Ventral aspect of regenerated limb three hundred days after amputation. The form of the toes is not typical. Pigment pads have developed on all toes and there is evidence of scattered masses of pad tissue along the post-axial margin near the base of the limb.

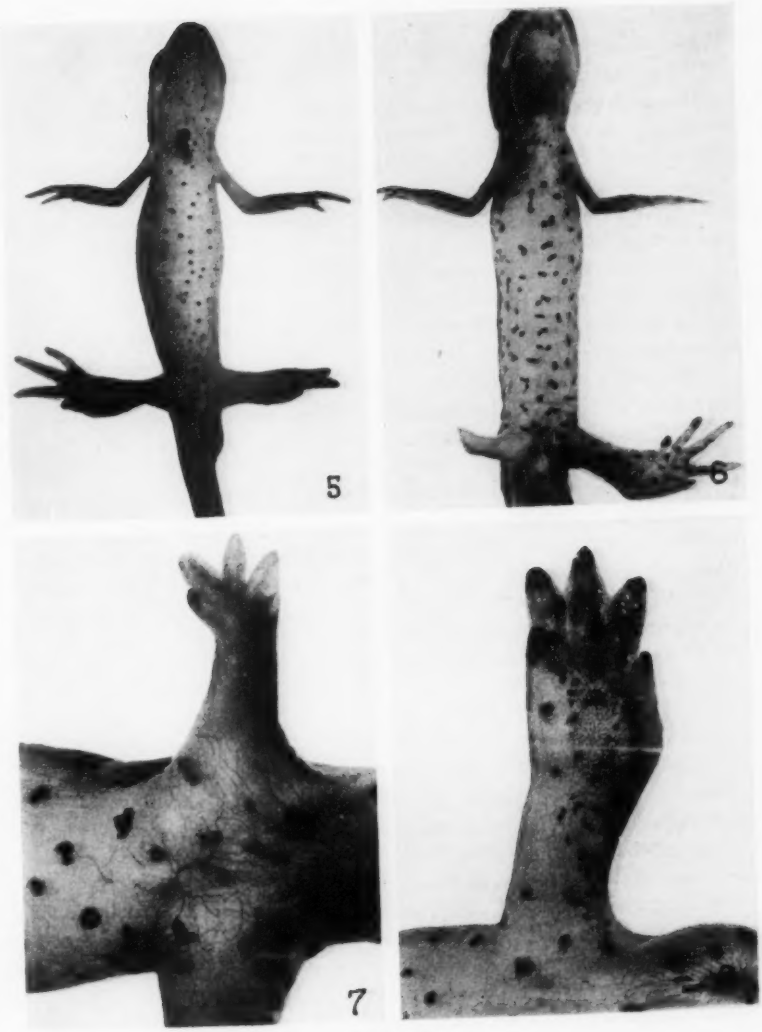

PLATE II

homoiotransplant in which the secondary sexual areas of the host were undisturbed and have given characteristic reactions following seven doses of pituitary extract. Photographic study at higher magnification (Fig. 4) of the pectoral graft shows complete absence of pigmented tissue.

Both auto- and homoiotransplantations have been performed on a series of animals in which limb regeneration was being studied. Confirmation of the results reported above was obtained (Figs. 5 and 6), thus excluding the possibility that lack of persistence of homoiotransplants might be due to the presence of pad tissue in excess of the normal amount.

Femoral pads were never observed to regenerate following extirpation. Denuded femoral areas to which skin from the pectoral region was transplanted failed to organize nuptial pads after more than three hundred days (Fig. 1).

Following amputation, limbs were found to regenerate but in no case were they found to equal the normal limb length nor were the relative length and form of the toes typical. These results confirm those reported by Collins (1932).

During regeneration the nuptial tissue was found to appear first in the toe pads. A definite, pigmented area was observed on the first toe tip seventy days after amputation (Fig. 7). Toe pigmentation then proceeded serially in a post-axial direction. Definite pigmentation in the femoral area was first observed at the end of the three-hundredth day of limb regeneration (Fig. 8). It was not possible to determine whether this process extended proximo-distally or disto-proximally.

Summary

The femoral pads of the newt, *Triturus viridescens*, respond to pituitary extract stimulation by characteristic hypertrophy and pigmentation. Autoplastic transplants of pads were found to persist intact after more than three hundred days while homoioplastic transplants lost the capacity to exhibit normal reactions after sixty days. This is not a function of the total amount of pad tissue present. Regeneration of the femoral pads in denuded areas was never observed. Regenerated limbs showed pigmentation of the first toe within seventy days. Evidence of the reorganization of the femoral pad was noted three hundred days after amputation.

It is a pleasure to acknowledge the helpful advice generously given by Dr. A. B. Dawson and Dr. H. W. Rand.

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OBSERVATIONS ON AN ENDAMŒBA PARASITIZING OPALINID CILIATES

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Introduction

Endamœba is well known as a parasite of Metazoa, but it had not been identified as a parasite of Protozoa until 1933, when a brief notice of this remarkable phenomenon was published by Stabler. The protozoans found infected with *Endamœba* were opalinid ciliates, living in the rectum of frogs and toads.

The first observers of endamœbæ within the opalinids either did not recognize them as parasites at all or identified them incorrectly. Thus, Metcalf (1923) described them as "secondary" and degenerating nuclei which he believed to be "abnormal" and "associated with parasitism." Carini (1933*a* and *b*) recognized them as parasites, but thought they represented a new genus which he called *Brumptina*, in honor of Professor Brumpt, who had seen but not identified the same objects in 1913. Since Stabler (1933) recognized these "secondary nuclei" and "*Brumptina*" as endamœbæ, the report has been confirmed by the recent observations of Carini and Reichenow (1935).

In the present contribution, we report further observations on the endamœbæ infecting opalinids from a number of different localities. These observations have been supplemented by independent examination of the original preparations of Metcalf and Carini.

Material and Methods

The material consists of the following opalinids parasitized by amœbæ: *Zelleriella hirsuta* in *Bufo cognatus cognatus* from Flagstaff, Arizona; *Z. opisthocarya* in *B. marinus* from Panama City, Panama; zelleriellas in *B. woodhousii* from Somerton, Arizona and from the vicinity of Tuba City, Arizona; and zelleriellas in *Pleurodema bibroni* from the vicinity of Coquimbo, Chile. A number of other species of anurans and their opalinids from different localities were also examined, but no endamœbæ were found in them.

The ciliates containing the parasites were studied to some extent in 0.8 per cent NaCl and in dilute Lugol's iodine solution, but mainly

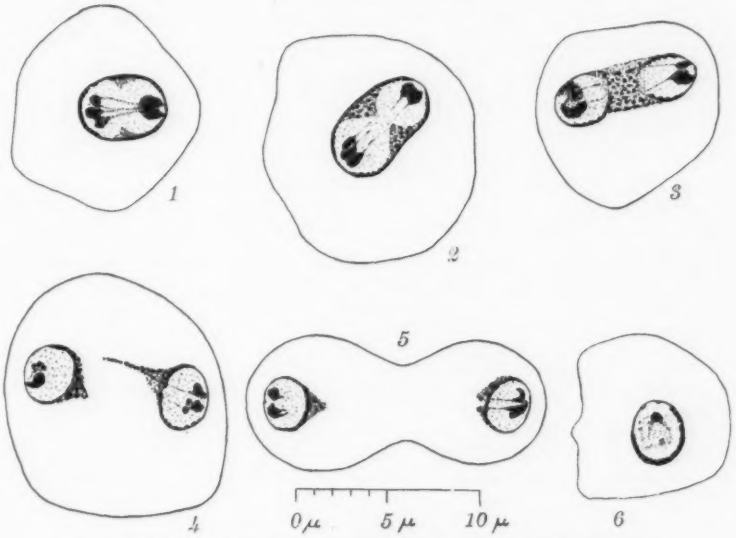
on smears fixed in warm Schaudinn's fluid containing 5 per cent of glacial acetic acid and subsequently stained in Heidenhain's hæmatoxylin. Some of the destaining was carried out in iron alum, some in a saturated aqueous solution of picric acid.

All drawings were made with the aid of the camera lucida.

Original Observations

General Description

The organism found living in the cytoplasm of these opalinids is an *Endamœba*, with all the features characteristic of the genus. It greatly resembles *Endamœba ranarum* morphologically.



Figs. 1–6. Late division stages in the endamœbæ. For explanation see text.

Observed in the living condition, the amœbæ appear as small refractile bodies within the cytoplasm of the actively moving ciliates. Due to their small size, they could easily be overlooked, as the opalinids turn and dart about. In the iodine solution, however, they are easily discernible and their *Endamœba* characteristics readily noted. The amœbæ seem to have little or no effect on the ciliate. Not only do the ciliates move actively about, but they also undergo normal binary fission despite the presence of numbers of amœbæ in their cytoplasm (Fig. 22).

Although a good number of species of anurans and their opalinids from different localities have been examined, only certain species of anurans from some localities and a certain percentage of these latter individuals contain ciliates parasitized by the endamœbæ. Within an individual anuran host, if the opalinids are thus parasitized, a number of these ciliates may still be entirely free from the amœbæ, some harboring a single amœba, others a number of them (Fig. 23) and still others may have their cytoplasm filled with well over a hundred of these parasites (Fig. 24).

The amœbæ are often seen lying in pockets in the cytoplasm of the ciliates and occasionally two or more amœbæ are found in the same pocket (Fig. 10). The space between the amœbæ and the cytoplasm may represent, partially at least, a shrinkage space resulting from fixation and dehydration. A number of amœbæ have been found exerting such pressure on the nuclear membranes of the opalinids that the membranes were markedly indented (Fig. 8). No amœba has ever been observed within a nucleus, however. Usually all the amœbæ in any one ciliate are in the same stage of development, i.e. all cysts or all trophozoites (Figs. 22, 23, and 24).

DESCRIPTION OF PLATES

Drawings all made with the aid of the camera lucida from material stained in Heidenhain's hæmatoxylin and destained in iron alum except where noted.

Explanation of Plate I

Figs. 7–18 are of the *Endamœba* living in the opalinids.

Figs. 19–21 are of *Endamœba ranarum*.

Fig. 7. Trophozoite in the periphery of *Zelleriella* showing ingested endospherules.

Fig. 8. An amœba indenting the nuclear membrane of the ciliate.

Fig. 9. Trophozoite, the nucleus of which shows an abundance of stainable material about the karyosome. Destained in a saturated aqueous solution of picric acid.

Fig. 10. Two amœbæ in the same pocket.

Fig. 11. Two nuclei in the same amœba.

Fig. 12. An amœba outside the opalinids. Note absence of food bodies and the abundance of stainable material in the center of the nucleus. Destained in picric acid.

Fig. 13. Amœba containing the *Sphærita*-like organisms. Destained in picric acid.

Fig. 14. Trophozoite showing the typical *Endamœba* features.

Fig. 15. Typical uninucleate cyst within a *Zelleriella*.

Fig. 16. A very small trophozoite. Destained in picric acid.

Fig. 17. Quadrinucleate cyst outside the ciliates. Note the *E. coli*-like chromatoids.

Fig. 18. Quadrinucleate cyst outside the ciliates. Note the *E. histolytica*-like chromatoid. Destained in picric acid. Compare with Fig. 21.

Fig. 19. *E. ranarum* trophozoite. Note ingested bacteria.

Fig. 20. Uninucleate cyst of *E. ranarum*.

Fig. 21. Quadrinucleate cyst of *E. ranarum*. Compare with Fig. 18.

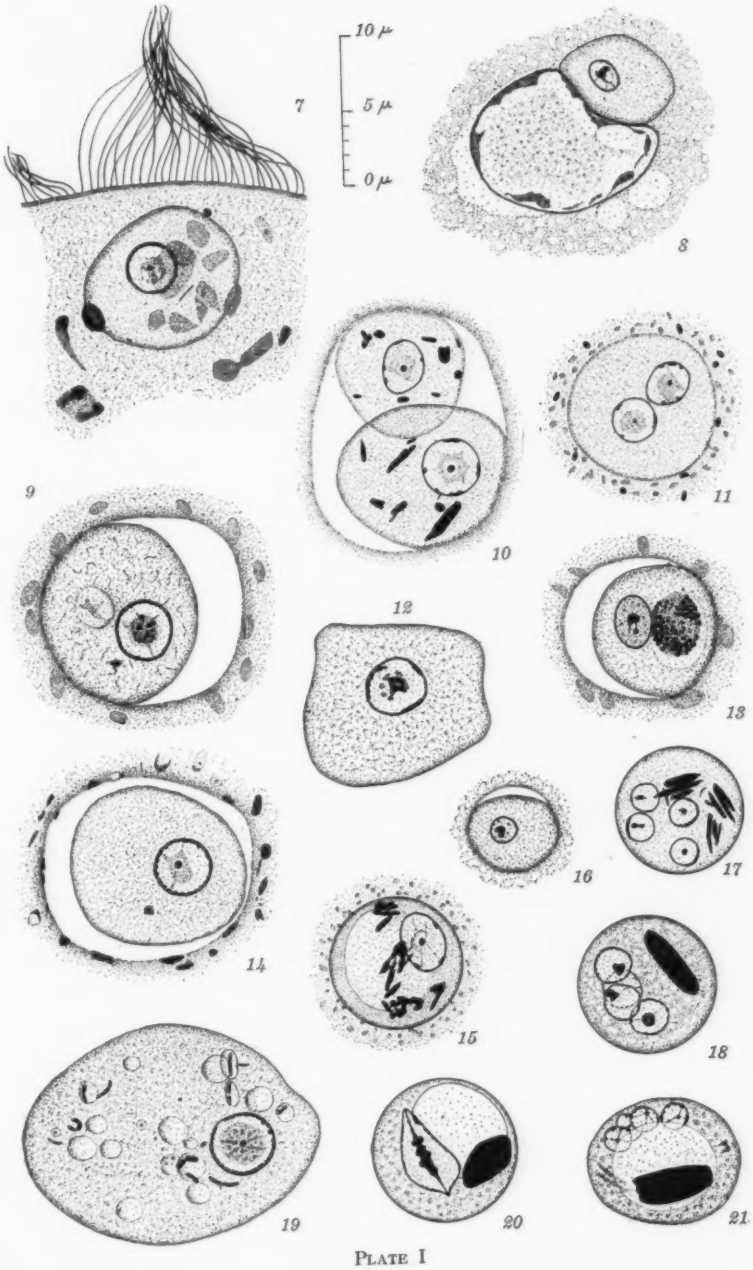
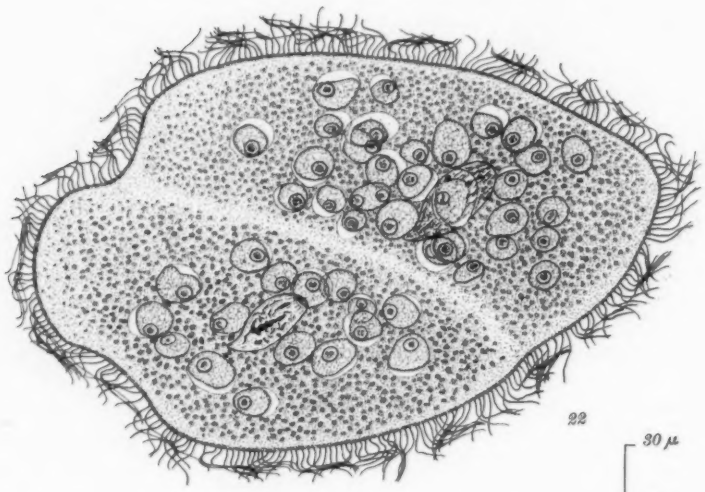
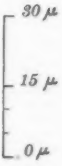


PLATE I





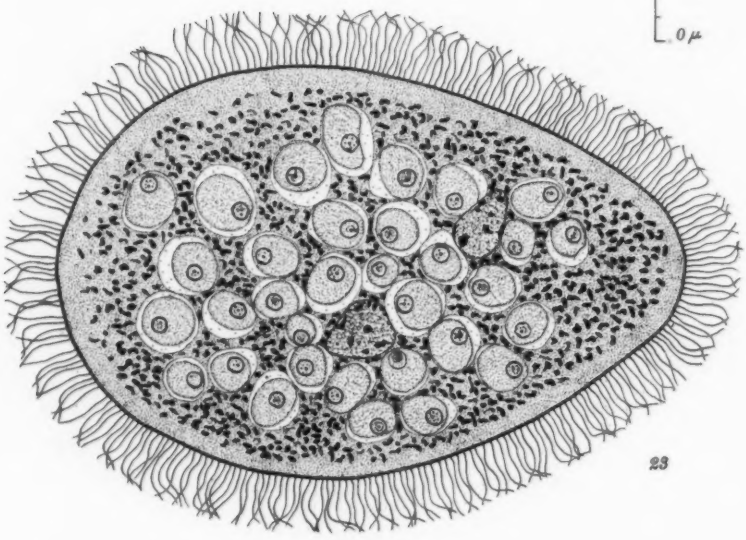


PLATE II

FIG. 22. A *Zelleriella* in a late stage of binary fission, containing many endamœbæ. Cilia diagrammatic.

FIG. 23. A *Zelleriella* fairly well filled with the trophozoites of the *Endamœba*. Cilia diagrammatic.

Although there may be these hundreds of amœbæ within the ciliates, relatively few are found free in the lumen of the rectum of the anuran host (Fig. 12). These latter are thought to represent amœbæ that have either escaped from, or been freed by the disintegration of the ciliates.

Trophozoites

The amœba is a fairly small one; seventy-five nearly spherical trophozoites taken at random from a number of different hosts range in diameter from 5.3 μ–14.3 μ, averaging 8.0 μ. Some ciliates contain enormous numbers of very small amœbæ only, while others harbor the large forms.

The amœbæ present typical *Endamœba* nuclei. In well-fixed material there is usually a fairly even ring of peripheral chromatin at the nuclear membrane (Figs. 7, 9, and 14). At times, however, this ring is broken into masses and blobs (Figs. 10, 11, and 12). A karyosome, which frequently appears as a discrete granule surrounded by an area of more lightly staining material (Figs. 7, 10, 11, and 14), shows particularly well when the stain has been differentiated in iron alum. After picric acid destaining this karyosome is usually less prominent and the surrounding material more deeply stained (Figs. 9 and 12). Occasionally two nuclei are found within the same amœba (Fig. 11).

A number of dividing stages have been observed in the trophozoites. However, only the later stages of mitosis have been recognized, an anaphase being the first stage of which we are certain (Fig. 1). In these later mitotic stages, in the mid-region of the elongating nucleus, there appears to be the progression of a series of granules from the nuclear membrane inward (Fig. 1). These granules do not appear to contribute to the formation of the chromosomes. As the daughter nuclei separate, this area of granules becomes larger (Figs. 2 and 3) and is finally stretched out as a "tail" from each of the telophase nuclei (Figs. 4 and 5). These granules finally disappear entirely and the reorganizing nucleus is featured by the layer of heavier peripheral chromatin on the one side of the membrane and the decidedly scarcer chromatin on the other (Fig. 6). This distribution of peripheral chromatin is typical of all the late telophase nuclei. It never entirely disappears from the nuclear membrane during mitosis. The exact chromosome number could not be determined from our material.

The question as to the food habits of these amœbæ is a difficult one to answer. They apparently feed on the endospherules of the opalinids, since these are the only inclusions noted in the trophozoites (Fig. 7). The apparently identical amœbæ found outside the ciliates contain no solid food.

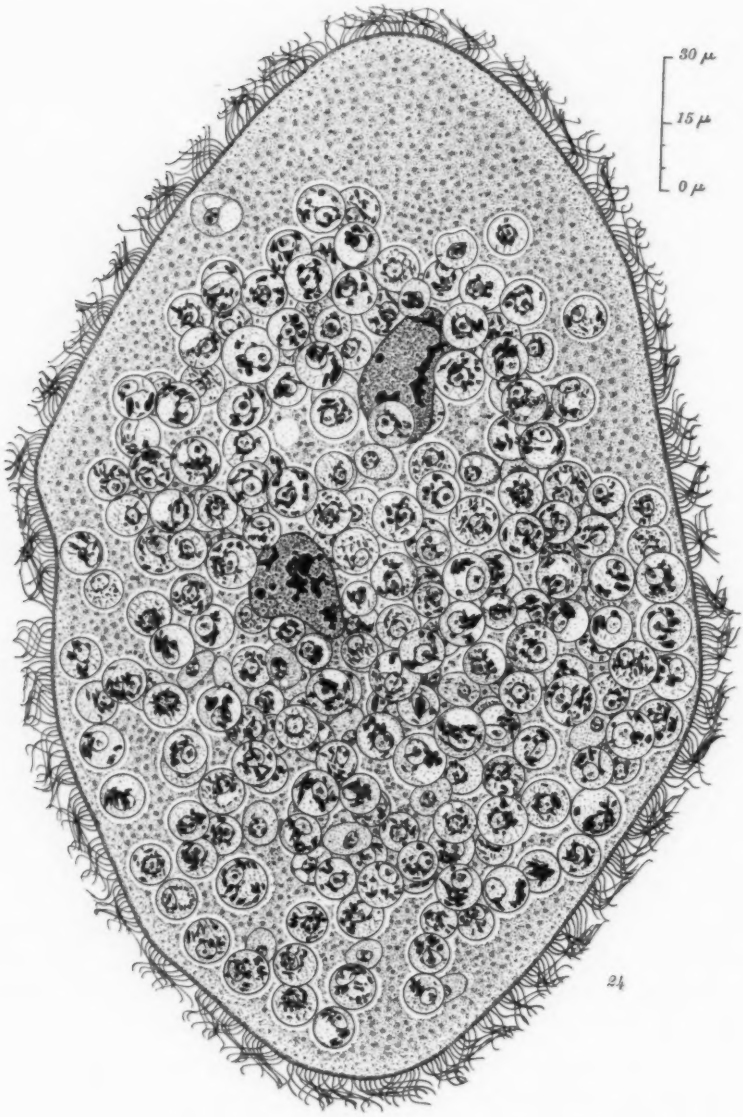


PLATE III

FIG. 24. *Zelleriella opisthocarya* containing over two hundred cysts of the *Endamœba*. Note uninucleate condition of the cysts, type of chromatoids, etc. Cilia diagrammatic.

Cysts

The cysts are less variable in size and slightly larger on the average than the trophozoites, though some trophozoites are larger than any cysts. Seventy-five cysts measured, range from 7.7 μ–10.8 μ, with an average diameter of 9.4 μ. Only the cysts within the ciliates were measured.

One of the most interesting facts concerning the cysts of this amœba is that, in our material, no single cyst in any ciliate ever contains more than one nucleus. In one opalinid, there are over two hundred cysts, all uninucleate (Fig. 24) and the only cases of more than one nucleus per cyst occur in those outside the ciliates (Figs. 17 and 18). Although division stages have been observed in the trophozoites, our material shows no cyst the nucleus of which is in any phase of mitosis.

TABLE I

Showing percentage infection of opalinids harboring the endamœbæ

Anuran host	Locality	Opalinid infected	Percentage infected in some individual anurans
Bufo cognatus cognatus	Flagstaff, Arizona	*Zelleriella hirsuta*	84
Bufo woodhousii	Near Tuba City, Arizona	*Zelleriella sp.*	19
Bufo woodhousii	Somerton, Arizona	*Zelleriella sp.*	22
Pleurodema bibroni	Near Coquimbo, Chile	*Zelleriella sp.*	91
Bufo marinus	Panama City, Panama	*Zelleriella opisthocarya*	51

The chromatoids of this amœba vary in appearance. Many are splinter-shaped, typical of *E. coli* (Figs. 15, 17, and 24), while others are bar-shaped, typical of *E. histolytica* (Fig. 18). Glycogen vacuoles are present in many of the cysts (Fig. 15).

Percentage Infection within Some Individual Anurans

Table I shows the percentage infection of opalinids harboring the endamœbæ. It is noted that in the 51 per cent infection of *Zelleriella opisthocarya* from *Bufo marinus* from Panama, only 4 per cent of the small and possibly precystic ciliates (averaging 60 μ in length) are

parasitized, whereas among the large forms (averaging 230 μ in length), 98 per cent are infected. A possible explanation of this interesting phenomenon is available. It is a well-known fact that, previous to encystment, there is a period in which the opalinids divide very rapidly, giving rise to very small forms which later encyst. It is possible that,

TABLE II

Showing anuran hosts, localities, etc. for opalinids known to harbor endamœbæ

Anuran host	Locality	Describer	Opalinid infected
Polypedates maculatus	Ceylon	Metcalf, 1923 (from Dobell's slide)	*Opalina virgula**
Bufo woodhousii	Utah	Metcalf, 1923	*Zelleriella sp.**
Bufo cognotus	Phoenix, Arizona	Metcalf, 1923	*Zelleriella hirsuta**
Paludicola signifera	City of São Paulo, Brazil	Carini, 1933	*Zelleriella brasiliensis*†
Engystoma ovale	City of São Paulo, Brazil	Carini, 1933	*Zelleriella falcata*‡
Bufo marinus	Panama City, Panama	Stabler, 1933	*Zelleriella opisthocarya*
Bufo woodhousii	Somerton, Arizona	Stabler, 1933	*Zelleriella sp.*
Leptodactylus ocellatus	Uruguay	Carini and Reichenow, 1935	*Zelleriella brasiliensis*
Bufo cognatus cognatus	Flagstaff, Arizona	Stabler and Chen, 1936	*Zelleriella hirsuta*
Bufo woodhousii	Near Tuba City, Arizona	Stabler and Chen, 1936	*Zelleriella sp.*
Pleurodema bibroni	Near Coquimbo, Chile	Stabler and Chen, 1936	*Zelleriella sp.*
Hyla nana	Angra dos Reis, State of Rio de Janeiro, Brazil	Stabler and Chen, 1936	*Zelleriella sp.*§
Bufo woodhousii	Utah	Stabler and Chen, 1936	*Opalina woodhousii*§

* Amœbæ described by Metcalf (1923) as "secondary" and degenerating nuclei.

† Changed by Carini and Reichenow (1935) to *Zelleriella paludicolæ*. Amœbæ described by Carini (1933) as *Brumptina brasiliensis*.

‡ Amœbæ described by Carini (1933) as *Brumptina paulista*.

§ From Metcalf's slides.

before encystment, the opalinids divide so fast that their rate of division is much more rapid than that of the amœbæ inside them. The large and parasitized zelleriellas which originally contain only a few amœbæ would produce, by successive divisions, a number of precystic forms, some with endamœbæ, others without.

Anuran Hosts Harboring Parasitized Opalinids and the Geographical Distribution of these Hosts

Metcalf (1923) figured *Opalina virgula* from *Polypedates maculatus* from Ceylon (Fig. 171*c*), a species of *Zelleriella* from *Bufo woodhousii* from Utah (Fig. 98*b*, *e* and *g*) and *Z. hirsuta* from *Bufo cognatus* from Arizona (Fig. 99*c*, *d* and *e;* Fig. 100*c*), containing what he called "secondary" and degenerating nuclei. The objects within those opalinids we now know, as the result of our study of Metcalf's original slides, to be identical with the endamœbæ described here. Carini (1933) described what he believed to be two species of organisms in zelleriellas from *Paludicola signifera* and *Engystoma ovale* from São Paulo, Brazil. These he was unable to identify and created for them the new genus *Brumptina*. These likewise prove to be identical with this *Endamœba* (Stabler, 1933; Carini and Reichenow, 1935). In a recent report on the subject, Carini and Reichenow (1935) have found the amœbæ in *Zelleriella brasiliensis* from *Leptodactylus ocellatus* from Uruguay. These, with the present authors' data, as shown in Table II, constitute all the observations that to our knowledge have been made on these amœbæ.

We have examined frogs and toads from such widely separated localities as Central and South America, Bermuda, the West Indies and a number of places in the United States. From some localities, large numbers of specimens of a given species of anuran have been examined. The same species of anuran host from different regions harbors opalinids infected with *Endamœba* in one locality and not in another. Then, too, from any particular region some individuals of a particular species of anuran contain parasitized opalinids, others do not.

Parasites of the Endamœbæ

In *Pleurodema bibroni* from Chile and *Bufo marinus* from Panama, some of the endamœbæ in the zelleriellas are parasitized by what appears to be a *Sphærita* (Fig. 13). These organisms are small and spherical, from one to fifty occurring in a single amœba. Occasionally they are found in the cytoplasm of the zelleriellas.

Discussion

It seems surprising that, with the exception of the short account by Stabler (1933) and the recent publication by Carini and Reichenow (1935), no other description of this organism has been published in which its true nature has been appreciated.

Carini and Reichenow are correct in believing Metcalf's *Zelleriella hirsuta* from *Bufo cognatus* to contain endamœbæ, but are mistaken in

suggesting that the objects in *Zelleriella sp.* from *B. woodhousii* are *Sphærita*. They overlooked Metcalf's figure of the same bodies in *Opalina virgula* (Metcalf, 1923, Fig. 171*c*).

From our examination of Carini's preparations, we can agree with Carini and Reichenow's (1935) assertion that the *Zelleriella* from *Paludicola signifera* contains an *Endamœba*, an opinion recorded by Stabler in 1933. Carini (1933) probably failed to recognize these objects as amœbæ because of poor fixation and staining, but we have found undoubted trophozoites (Fig. 30) and cysts (Fig. 31) within the zelleriellas from his slides.

Unlike our own, Carini's preparations of opalinids from *P. signifera* show an abundance of uni-, bi- and quadri-nucleate cysts free from the ciliates (Figs. 32, 33, 34 and 35). As mentioned by Carini and Reichenow, these cysts present essentially the same morphology as the ones within the ciliates. We have observed, however, an additional feature of these cysts in Carini's preparations not noted by the above workers. The majority of the cysts outside the opalinids possess an enveloping membrane outside of and apart from the cyst wall (Figs. 32, 33, 34, and 35). This was also noted as a feature of *E. ranarum* by Sanders in 1931.

Concerning the trophozoites, Carini and Reichenow (1935) state that, with one exception, they were never found free from the ciliates. In Carini's own material from *P. signifera* the present writers have

EXPLANATION OF PLATE IV

FIG. 25. *Endamœba* trophozoite in *Zelleriella hirsuta* from *Bufo cognatus*, from Metcalf's slide.

FIG. 26. *Endamœba* trophozoite in *Zelleriella sp.* from *Bufo woodhousii*, from Metcalf's slide. Due probably to unsatisfactory technique, the *Endamœba* characteristics are not well shown in this animal.

FIG. 27. *Endamœba* trophozoite in *Opalina woodhousii* from *Bufo woodhousii*, from Metcalf's slide.

FIG. 28. *Endamœba* trophozoite in *Zelleriella sp.* from *Hyla nana*, from Metcalf's slide.

FIG. 29. *Endamœba* trophozoite in *Opalina virgula* from *Polypedates maculatus*, from Dobell's slide.

FIG. 30. *Endamœba* trophozoite in *Zelleriella paludicolæ* from *Paludicola signifera*, from Carini's slide.

FIG. 31. *Endamœba* cyst in *Zelleriella paludicolæ* from *Paludicola signifera*, from Carini's slide. Compare with Fig. 32.

FIG. 32. *Endamœba* cyst outside the ciliates from *Paludicola signifera*, from Carini's slide. Note the single nucleus and extracystic membrane. Compare with Fig. 31.

FIG. 33. *Endamœba* cyst outside the ciliates from *Paludicola signifera*, from Carini's slide. Note the four nuclei and extracystic membrane.

FIG. 34. Same as Fig. 33.

FIG. 35. *Endamœba* cyst outside the ciliates from *Paludicola signifera*, from Carini's slide. Note the two nuclei and extracystic membrane.

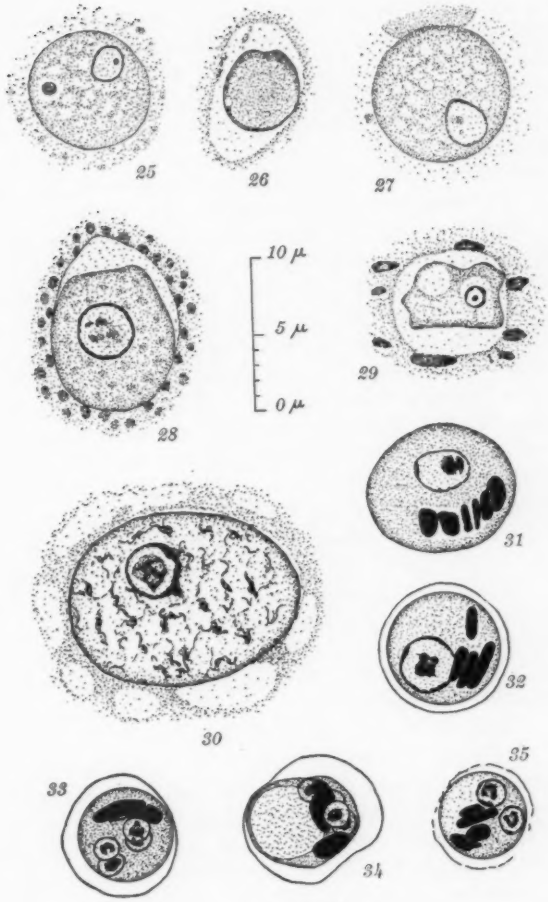


PLATE IV

observed dozens of amœbæ outside the opalinids. We also cannot support Carini and Reichenow's assertion that no karyosomes can be seen in the nuclei of these amœbæ since this nuclear structure has been found in our material. Then, too, in our slides the amœbæ inside the ciliates are occasionally found filled with the endospherules of the latter, in contrast to the above writers' findings that they were always free of solid food.

Carini and Reichenow believed that, in their material, trophozoites of the amœba invade ciliates already containing the uninucleate cysts and then proceed to phagocytize these cysts. Their figure of this is unconvincing and we have seen nothing in any of Carini's nor in our own slides suggesting this phenomenon.

This brings us to the question of the specific name for this amœba. Carini and Reichenow believed it to be either identical with *E. ranarum* or a race or species derived from it. Stabler (1933) identified it as an *Endamœba*, a decision confirmed by Carini and Reichenow (1935). He gave it the specific name *E. brasiliensis*, since Carini (1933), who gave the first description of these organisms, has designated *B. brasiliensis* as the name of his first species of *Brumptina* (= *Endamœba*). It has since been recalled that Aragão in 1914 used the name *E. brasiliensis* for what he considered to be a new amœba from man. It is generally agreed that Aragão's description deals mainly with *Endamœba coli*, the name therefore being dismissed as a synonym of the latter form. *E. brasiliensis* cannot, then, be revived for the form described here and as Carini designated a second species of *Brumptina*, *B. paulista*, this species name becomes the one available. If this is a distinct species of amœba, its name should then be *Endamœba paulista* (Carini, 1933).

However, at the present time we do not attempt to establish its specific identity, because the question as to its synonymy with *E. ranarum* is still unanswered. It must be admitted that morphologically they are quite similar, presenting the extracystic membrane, rather similar chromatoids, etc. *E. ranarum*, however, is somewhat larger, most writers (Sanders, 1931) stating that it ranges from 10 μ –30 μ in diameter, while the range for the amœba from the opalinids is 5.3 μ–14.3 μ. Also the food of the latter organism seems to be different. *E. ranarum* feeds readily on bacteria and debris in the rectum of the anuran, while these amœbæ, outside the ciliates, are never observed to contain food and those in the ciliates contain ingested endospherules only. In all probability infection experiments will be necessary to finally establish its identity.

Still another question concerns the mode of entrance of the amœbæ

into the astomatous opalinids. This process has never been observed by us. It seems probable that one or a few amœbæ may enter a ciliate and the progeny eventually fill the host. They may enter the ciliates when the rectal contents of the frogs and toads are rather dry, this condition preventing freedom of motion on the part of the ciliates. As it has been shown that the opalinids can divide successfully when containing amœbæ and that division stages have been observed in the amœbæ within the ciliates, these, too, may be important factors in carrying on the infection.

It seems possible that the cysts of the endamœbæ which have been freed from the ciliates constitute an infective stage so far as the new anuran host is concerned. They may be deposited in the ponds at the breeding season and be available for ingestion, along with the cysts of the opalinids, by the tadpoles. In the new anuran host we again face the problem of the penetration of the rapidly swimming ciliates by the slowly moving amœbæ. It is also possible that the amœbæ may even be within the encysted opalinids and pass from adult anurans to tadpoles with the latter.

Many other problems concerning this amœba remain yet to be solved. Why do some ciliates contain such large numbers of very small amœbæ and is there any significance to be attached to these small forms? Do the cysts always remain in the uninucleate condition until released from the ciliates? What are the appearances of the prophase and metaphase stages of mitosis? Would some races of undoubted *E. ranarum* invade opalinids?

Summary

1. Observations are presented on an *Endamœba* living in the opalinid ciliates from anuran hosts.

2. Large numbers of frogs and toads from widely separated regions have been examined. The endamœbæ have been found in some of the opalinids collected from the United States (Arizona and Utah), Panama (Panamá City), Brazil (states of São Paulo and Rio de Janeiro), Chile (near Coquimbo), Uruguay and Ceylon. In a given locality, only certain species of anurans and a certain percentage of individuals of a given species contain the parasitized opalinids.

3. Within some individual anuran hosts, 91 per cent of the ciliates are infected. There may be a single, a few, many, or over a hundred of these amœbæ within a single opalinid. Usually in any one ciliate all the amœbæ are in the same stage of development, either cysts or trophozoites.

4. The amœbæ seem to produce no serious effect on the opalinids,

as the latter swim actively about in the saline solution and were seen to undergo binary fission even though heavily parasitized.

5. The trophozoites range in diameter from 5.3 μ–14.3 μ, averaging 8.0 μ. They appear to feed on the endospherules of the opalinids and a number of dividing stages have also been observed in the trophozoites.

6. The cysts are less variable in size and are slightly larger on the average (9.4 μ) than the trophozoites, though some trophozoites are larger than any cysts. Only uninucleate cysts have been found within the opalinids although uninucleate, binucleate and quadrinucleate ones were found outside the ciliates. The chromatoids vary in appearance; many are splinter-shaped, while others are bar-shaped. Glycogen vacuoles are present in many cysts.

7. In the zelleriellas from *Pleurodema bibroni* from Chile and in *Bufo marinus* from Panama, some of the endamœbæ were invaded by a *Sphærita*-like organism, which was also observed in the cytoplasm of some of the ciliates.

Acknowledgments

This study was aided by a grant from the University of Pennsylvania Chapter of the Society of Sigma Xi and a grant from the Bache Fund, National Academy of Sciences, and was completed while one of us (T. T. C.) was holding a Sterling Research Fellowship at Yale University. We wish to express our sincerest gratitude to Dr. D. H. Wenrich for his constant interest and advice throughout the investigation.

For live specimens of Anura from Panama, Arizona, Chile, Bermuda and the West Indies, we are indebted to the following: Dr. H. C. Clark of the Gorgas Memorial Laboratory of Panama; Mr. L. M. Klauber of the Natural History Museum at San Diego, California; Dr. H. S. Colton of the Museum of Northern Arizona at Flagstaff, Arizona; Mr. M. G. Netting of the Carnegie Museum at Pittsburgh, Pennsylvania; Messrs. Rafael Varleta and Luis F. Varleta of La Serena, Chile; Dr. J. F. G. Wheeler of the Bermuda Biological Station; and to Messrs. K. V. DuQuesney and Charles DuQuesney of Highgate, Jamaica. For the loan of a number of slides of opalinids we wish to thank Dr. M. M. Metcalf, Dr. Robert Hegner of the Johns Hopkins University and Dr. R. W. Miner of the American Museum of Natural History. Also through the courtesy of Dr. Metcalf, we were able to examine some of the original preparations of Dr. A. Carini and Mr. C. C. Dobell, F.R.S.

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FURTHER STUDIES ON THE ENDAMŒBÆ PARASITIZING OPALINID CILIATES

TZE-TUAN CHEN AND ROBERT M. STABLER

(From the Osborn Zoölogical Laboratory, Yale University, and the Zoölogical Laboratory, University of Pennsylvania)

Introduction

In two previous papers (Stabler, 1933; Stabler and Chen, 1936) we have described in detail the morphology of both the cysts and the trophozoites of amœbæ parasitic in opalinids. In the present note, we wish to report additional genera and species of opalinids infected, the geographical distribution of the amœbæ, and a method of transmission of amœbæ from adult anurans to tadpoles. A summary of this paper has been published as an abstract (Chen and Stabler, 1935).

Material and Methods

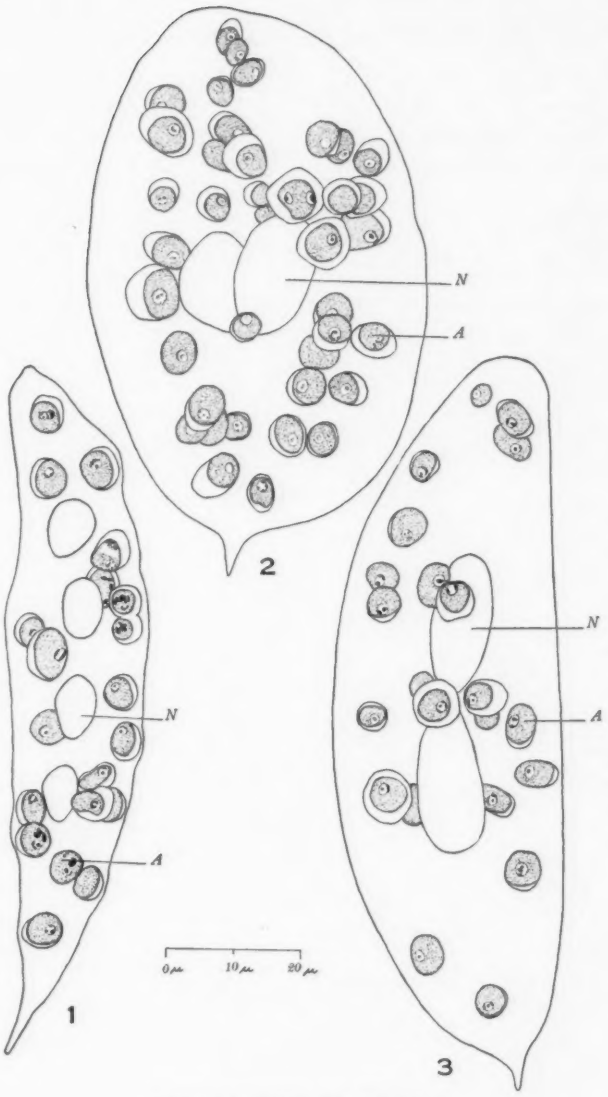
The present report includes observations on the following genera and species of opalinids parasitized by the amœbæ: *Protoopalina stevensoni*, *P. nutti* and *P. regularis*, all in *Bufo regularis regularis* from Cairo; *P. quadrinucleata* in *Rana plancyi* from Peiping; zelleriellas in *Bufo marinus* from several localities in the state of São Paulo; *Cepedea longa* in *Rana limnocharis* from Nanking; and *Opalina woodhousii* in *Bufo woodhousii* from Arizona. A number of other species of opalinids were also examined but found to be free from the parasites.

Most of the material was secured in the following way. The opalinids, after being smeared on the cover glasses, were immediately fixed in warm Schaudinn's fluid containing 5 per cent of glacial acetic acid. This was done in the localities where the different anuran hosts were found and the smears, after being gradually dehydrated, were preserved in 70 per cent alcohol and shipped to us. The ciliates were then stained in Heidenhain's hæmatoxylin, the destaining being carried out mainly in picric acid, although some smears were destained in iron alum.

All drawings were made with the aid of the camera lucida.

Observations and Discussion

Studies on additional material obtained from distant localities have revealed the fact that the endamœbæ living in the opalinids have a very wide geographical distribution. The animals have been found in some of the opalinids collected in Egypt (Cairo), China (Nanking



EXPLANATION OF PLATES

Drawings all made with the aid of the camera lucida from material stained in Heidenhain's hæmatoxylin. *N* = nucleus of the opalinid host, *A* = the parasitic amœba.

PLATE I

FIG. 1. Trophozoite of *Protoopalina quadrinucleata* containing many amœbæ.
FIG. 2. Trophozoite of *Protoopalina stevensoni* containing many amœbæ.
FIG. 3. Trophozoite of *Protoopalina nulti* containing many amœbæ.

PLATE II

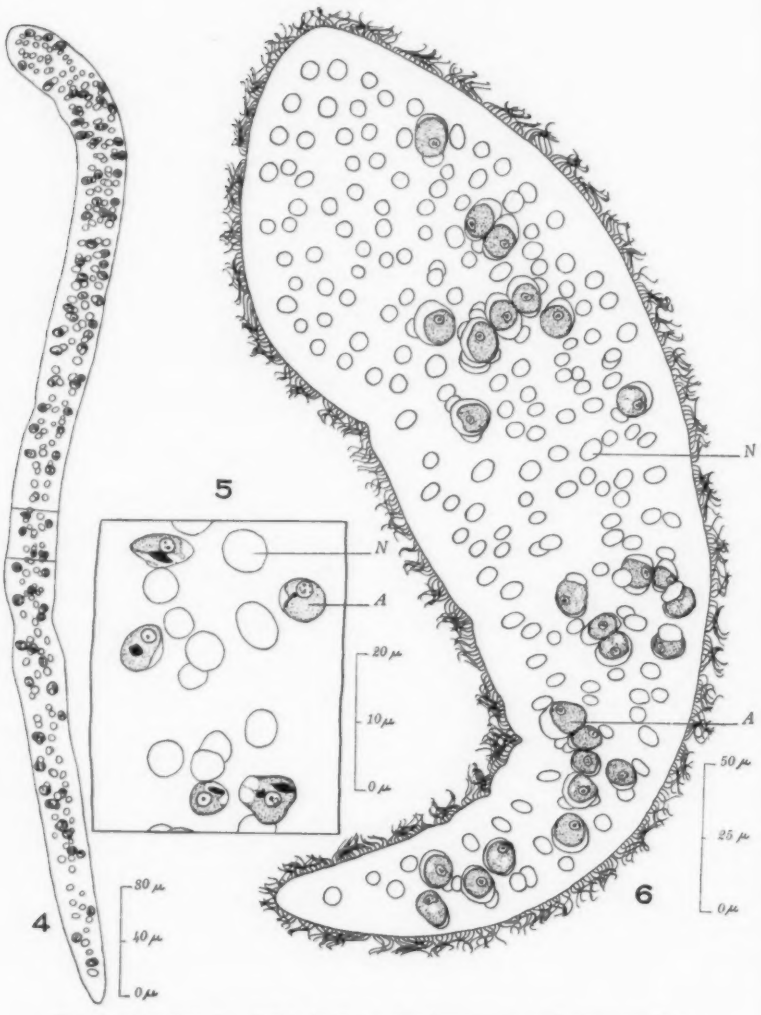


FIG. 4. Trophozoite of *Cepedea longa* showing presence of many amœbæ.
FIG. 5. Area from Fig. 4 drawn at a high magnification in order to show the details of the amœbæ.
FIG. 6. Trophozoite of *Opalina woodhousii* showing presence of numerous amœbæ. Cilia diagrammatic.

and Peiping), Ceylon, the United States (Utah and Arizona), Panama (Panama City), Brazil (the states of São Paulo and Rio de Janeiro), Uruguay and Chile (near Coquimbo). The localities cited in our previous paper (Stabler and Chen, 1936) are included in the present list.

The presence of endamœbæ in two genera of the Opalinidæ—*Zelleriella* and *Opalina*—has already been reported by us, but further studies have shown that different species belonging to all four genera of the family may be parasitized (Figs. 1–6). In the previous paper (Stabler and Chen, 1936) there were listed eight species of *Zelleriella* and two species of *Opalina* as being infected and in the present study,

TABLE I

Showing percentage of infection for opalinids harboring endamœbæ. (For a list of other opalinids infected and their percentage of infection, see Tables I and II, Stabler and Chen, 1936.)

Anuran host	Locality	Opalinid infected	Percentage infected in some individual anurans
Bufo regularis regularis	Cairo, Egypt	*Protoopalina stevensoni*	7
Bufo regularis regularis	Cairo, Egypt	*Protoopalina nutti*	39
Bufo regularis regularis	Cairo, Egypt	*Protoopalina regularis*	34
Rana plancyi	Peiping, China	*Protoopalina quadrinucleata*	2
Bufo marinus	Taubaté, State São Paulo, Brazil	*Zelleriella sp.*	100
Rana limnocharis	Nanking, China	*Cepedea longa*	*
Bufo woodhousii	Near Tuba City, Arizona	*Opalina woodhousii*	50

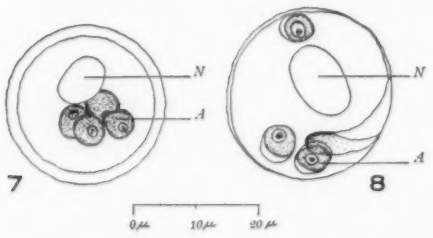
* Percentage infection in this form could not be determined accurately because of the small number of opalinids available.

four species of *Protoopalina*, one additional species of *Zelleriella*, and a species of *Cepedea* have been found to contain the parasites (Table I).

As noted before, in the different localities, only certain species of anurans and a certain percentage of individuals of a given species harbor the parasitized opalinids. For example, out of 37 frogs and toads (*Rana mascareniensis mascareniensis* and *Bufo regularis regularis*) examined at Cairo, 35 have been found to contain the opalinids. Among these 35 specimens, only five (all *Bufo regularis regularis*)

contain opalinids parasitized by amœbæ, whereas others are free from the parasites. There is a great range of variation in the percentage infection of opalinids among individual anuran hosts (Table I). The zelleriellas in some individuals of *Bufo marinus* from Taubaté, State of São Paulo, Brazil, are 100 per cent infected, whereas in *Rana plancyi* from Peiping only a few specimens of *Protoopalina quadrinucleata* contain any amœbæ. It is difficult to explain such an unusually low percentage of infection in this *Protoopalina*.

In some anuran hosts, when their opalinids are very heavily parasitized, the small, precystic forms also contain numbers of amœbæ. The encysted ciliates, when present in these anuran hosts, may also harbor the parasites. In the preparations of *Protoopalina nutti* in *Bufo regularis regularis* from Cairo and zelleriellas in *Bufo marinus* from Taubaté, amœbæ have been observed within the opalinid cysts (Figs. 7 and 8), lying in the cytoplasm of the ciliates. In the opalinid



FIGS. 7 and 8. Cysts of opalinids containing amœbæ.
FIG. 7. Cyst of *Zelleriella sp.* from *Bufo marinus* containing four amœbæ.
FIG. 8. Cyst of *Protoopalina nutti* containing three amœbæ.

cysts only trophozoites of the amœba have been found. Such parasitized encysted opalinids were particularly numerous in the *Bufo marinus* material; in one individual host nearly 100 per cent of the cysts were infected. These cysts contain from one to seven amœbæ, although the majority harbor only two, three, or four of them. On one slide, for example, out of 205 cysts taken at random, 67 contain 3 amœbæ per cyst, 65 contain 2, 32 contain 4, 23 contain only one, 15 contain 5, 2 contain 6, and only one cyst contains none.

It seems likely that the presence of amœbæ within the encysted opalinids constitutes an important method of transmission of amœbæ from adult anurans to tadpoles, thus establishing infections in the new generation of anurans. During the breeding season of frogs and toads, the opalinid cysts containing the amœbæ may be extruded and deposited in the ponds. The young tadpoles, browsing at the bottom

of the pond, may ingest the parasitized opalinid cysts together with diatoms, other algæ, and debris. The encysted opalinids (carrying the amœbæ in their cytoplasm), after reaching the intestines of the tadpoles, escape from the cysts and later develop into trophozoites, probably still containing the amœbæ. Thus the amœbic infection of the opalinids may become established in these young anurans.

Summary

1. The amœbæ parasitic in the opalinid ciliates have a very wide geographical distribution, being found in Egypt, China, Ceylon, the United States, Panama, Brazil, Uruguay and Chile.

2. Different species belonging to all the four genera of the family Opalinidæ—*Protoopalina*, *Zelleriella*, *Cepedea*, and *Opalina*—have been found parasitized by the amœbæ.

3. There is a great range of variation in the percentage infection of opalinids in different individual anuran hosts. In some hosts it may be as low as 2 per cent, whereas in some others 100 per cent of the opalinids may be infected.

4. The amœbæ have been found within numerous opalinid cysts. Each cyst may contain one to seven amœbæ although the majority of cysts harbor only two, three, or four of them. The presence of amœbæ in the encysted opalinids probably constitutes an important method of transmission of amœbæ from adult anurans to tadpoles.

Acknowledgments

This study was aided by a grant from the Bache Fund, National Academy of Sciences, and was carried on while one of us (T. T. C.) was holding a Sterling Research Fellowship at Yale University. We are also indebted to Dr. D. H. Wenrich for his helpful advice. We wish to express our thanks to Professor H. Sandon, formerly of the Egyptian University at Cairo, Professor J. C. Li, and Mr. C. S. Lin of Yenching University at Peiping, and Professor S. B. Pessoa of Faculdade de Medecina de São Paulo, Brazil, for large numbers of well-fixed smears of opalinids from these different localities; and to Mr. Da-Shu Nie of the Science Society of China at Nanking for the loan of a number of slides.

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THE EARLY DEVELOPMENT OF ASCIDIAN EGGS

ARTHUR COHEN[1] AND N. J. BERRILL

(From the Department of Zoölogy, McGill University, Montreal)

Introduction

The eggs of ascidians have been a favorite material for investigation for many years. Owing to the presence of extra-oval membranes, observations on the nude egg were not possible. This difficulty was finally overcome by the method of Berrill (1932), who observed that hatching was partially effected by the digestion of the membranes and experimented to discover a readily accessible source of suitable enzymes. Effective enzymes he found were contained in the stomach juices of certain Crustacea, for example the crab, *Munida*. As a result of this discovery, it is now possible to obtain any desired quantity of nude eggs.

Materials and Methods

The observations described below were made on the egg of the ascidian, *Ascidiella aspersa*, obtained in the vicinity of Plymouth, either at Mill Bay Docks or at the Salstone (Salcombe) and *Phallusia mammillata* obtained at the Salstone during the months of May and June, 1935. As soon as was possible the ascidians were placed in basins through which sea water was circulated. Under these conditions eggs capable of producing larvæ could be obtained for at least several days. Of the stains used—Clark-Lubs sulphthalein group, Nile Blue Hydrochloride and Neutral Red—only the latter two were found able to penetrate the egg. The Clark-Lubs group will not penetrate even after several hours, nor will they penetrate when the medium is changed to isotonic NaCl solution.

To obtain the eggs the integuments overlying the oviduct were removed carefully with a small pair of forceps, the oviduct punctured with a sterile steel needle, the eggs withdrawn with a pipette and placed at once in a small glass dish containing sterile filtered sea water. The egg membranes were then removed with the stomach juice of the crab

[1] The observations recorded here were made at the Laboratory of the Marine Biological Association of the United Kingdom, while holding the Moyse Travelling Scholarship of McGill University. I wish to express my gratitude to Professor J. Stanley Gardiner, F. R. S., for his kind permission to occupy the Cambridge Table, and to Dr. E. J. Allen, F. R. S., and the staff of the Laboratory for their willing and courteous coöperation at all times.

Munida, according to the method described by Berrill (1932). With a dilution of one part *Munida* stomach juice to fifty parts sea water the membranes were readily digested off and the previously floating eggs then sink to the bottom of the dish. The resulting nude eggs when washed with fresh sea water were capable of giving perfect development.

The stains were made up in 0.5 per cent concentration in distilled water. About one drop was sufficient to give a faint coloration to 15–20 cc. of sea water, into which the nude eggs were then introduced. Ten to fifteen minutes were sufficient to give the egg a distinct and characteristic stained appearance. The eggs were then transferred to fresh sea water; they retained the stain throughout the development of the egg.

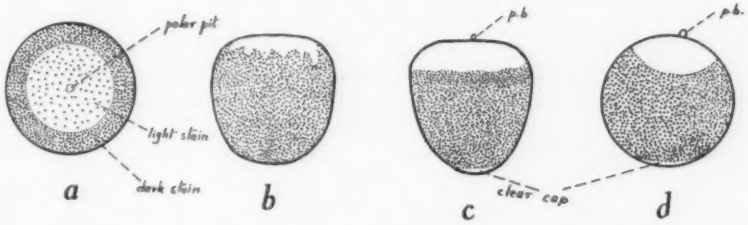

FIG. 1. Nude egg of *Ascidiella aspersa* vitally stained with Nile Blue. The dots indicate the limits and density of the stain. (*a*) unfertilized egg; (*b*) 18 minutes after insemination; (*c*) 21 minutes after insemination—the first polar body has been extruded; (*d*) 24 minutes after insemination, the egg has returned to a spherical condition.

Sperm was obtained directly from the spermduct in a manner similar to that described above for obtaining the eggs. Fertilization was effected by adding the sperm to the dish containing the stained eggs. After about five minutes the eggs were washed free from sperm.

A binocular dissecting microscope was used and when necessary a camera lucida. All observations recorded were made on the living egg.

BEHAVIOUR OF STAIN FROM FERTILIZATION TO FIRST POLAR BODY

The appearance of a stained unfertilized egg of *Ascidiella aspersa* is shown in Fig. 1, *a*. There is present a peripheral deeply-stained layer extending inward for about one-third of an egg radius. The central part of the egg stains much less intensely and is well marked off from the peripheral zone. This is even more strikingly evident in

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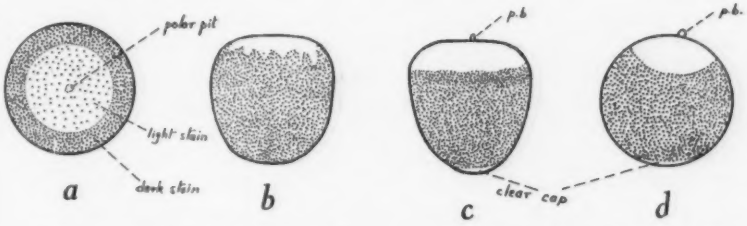

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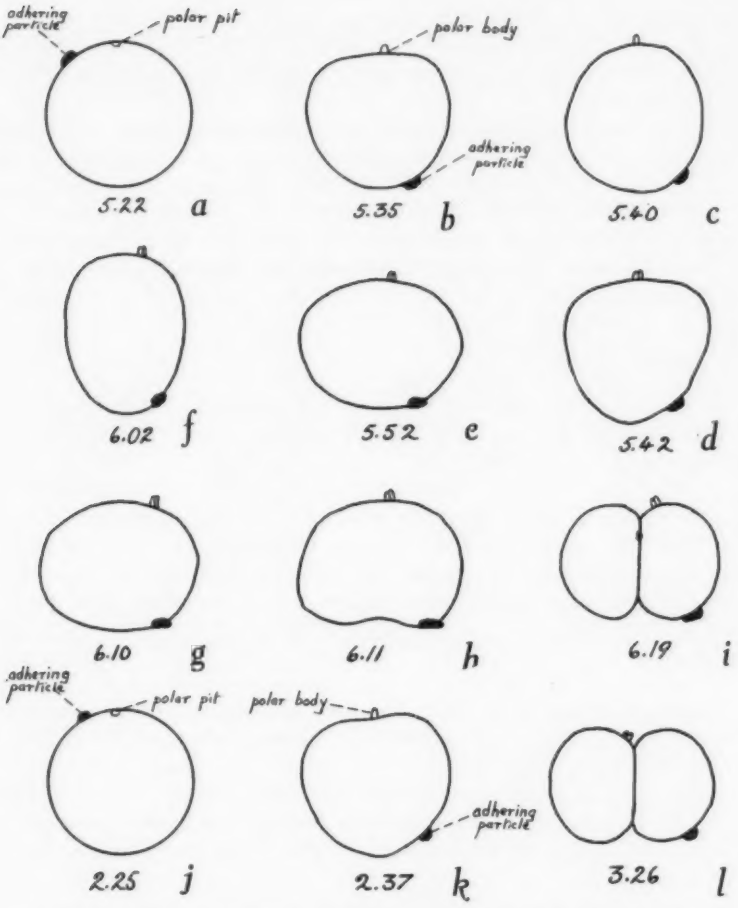

FIG. 2. Nude eggs of *Ascidiella aspersa* following fertilization showing the relationship between an adhering particle and the polar-pit, and the same particle to the first polar body. The figures indicate the time in hours and minutes of the observations. (*a*)–(*i*) records of one egg; (*j*)–(*l*) records of a second egg.

the egg of *Phallusia*. The egg presents the same appearance from all aspects with but one exception. This is the presence of a minute, unstained area, the "polar pit" ("la tache polaire," Dalcq, 1932), on the periphery of the egg, which shows up clearly against the stained background. About 18 minutes after insemination the egg loses its spherical shape and becomes somewhat flattened in the region of the "polar pit." At the same time the stain disappears from the region of the egg just beneath the flattened part (Fig. 1, *b*). The flattening and loss of stain occur very rapidly, requiring only a few seconds. Coincident with these changes the stain in the remainder of the egg no longer remains distinctly separated into peripheral and central regions but assumes a homogeneous distribution. About two minutes after these changes the first polar body is cut off from the clear flattened area. The egg has become somewhat elongated in what can now be identified as the animal-vegetal axis (Fig. 1, *c*). At the same time it can be seen that there is present a thin peripheral layer in the vegetal pole which is clear and unstained. From its position this clear cap seems to correspond to the "milky or yellowish crescent" observed by Tung (1932) on this egg, and possibly to the yellow crescent in the egg of *Styela partita* (Conklin, 1905). A few minutes after the polar body is extruded the egg returns to a spherical condition and the stained and unstained areas are clearly seen (Fig. 1, *d*).

Determination of the Site of Polar Body Formation

Dalcq (1932) has described the "polar pit" as the site of formation of the second polar body, and adds in a footnote (p. 229) that he has never observed more than one polar body ("globule") cut off. The latter statement is contrary to the present authors' observations, for numerous eggs were observed with two or even three polar bodies present, the latter condition no doubt indicating that the first polar body had divided. However, the polar bodies are very readily knocked off when the egg is manipulated and it is only rarely that all three polar bodies are seen on the egg. Furthermore, the method employed by Dalcq to remove the egg membranes, by the use of finely pointed forceps (p. 227, *op. cit.*), is doubtless a tedious and laborious method so that the number of eggs investigated by this author was probably small, whereas by the method of digesting off the membranes as was used in the present investigation, hundreds of nude eggs were easily obtained for observation. Nor was it possible to confirm the statement that the polar-pit is the site of the second polar body (or of the first). As was mentioned above, the area containing the unstained polar pit loses its stain shortly *before* the polar body is cut off

so that it was not possible to observe the relationship between polar-pit and polar-body directly. However, if the relationship between the polar-pit and some fixed point on the egg could be determined before the polar-body were formed, and this same fixed point compared with the newly-formed polar-body, the polar-pit and polar-body should both bear the same relationship to the fixed point on the egg, if there is an identity of site between them. This was tested in the following manner.

Unfertilized nude eggs of *Ascidiella aspersa* are stained with Nile Blue. After washing the eggs, sperm was added, about five minutes allowed to elapse, the eggs washed free from sperm and placed in a dish under a binocular microscope. Then it can be seen that particles have adhered to some of the eggs. An egg to which a suitable particle has become attached is selected and kept in the centre of the field, and a camera lucida drawing is made showing the position of the adhering particle and the polar-pit. Upon the appearance of the first polar-body another drawing is made to show the position of the particle and the polar-body, and the drawing compared with the previous one. Figure 2, *a–i* shows a series of drawings of the same egg from five minutes after insemination to the first cleavage. Inspection of these drawings reveals at once that the small angle formed by lines joining the adhering particle and polar-pit to the centre of the egg does not compare with the considerably larger angle formed by the lines joining the particle and polar-body to the centre of the egg. Figure 2, *j–l* shows another egg in which again the above-mentioned condition obtains. Observations on several other eggs gave similar results. In one case the angle before and after polar-body formation was approximately the same, but this is to be expected occasionally if, as the above drawings suggest, there is no relationship between the site of the polar-pit and the polar-body.

Now, as will be described below, the egg undergoes a series of changes in shape from just before the formation of the polar-body to the time of first cleavage (Fig. 3). It might be expected, then, that the apparent change in the angle before and after polar-body formation was due to movement of the particle over the surface of the egg due to movement of the surface layer of the egg to which it adheres. But this is hardly likely to be the case, since the change in shape which the egg undergoes before polar-body formation is a simple flattening and elongation, whereas from the time of polar-body formation to first cleavage the egg undergoes many and varied changes, yet during this period the relationship between particle and polar-body remains the same, as is seen from Fig. 2, *c–i*.

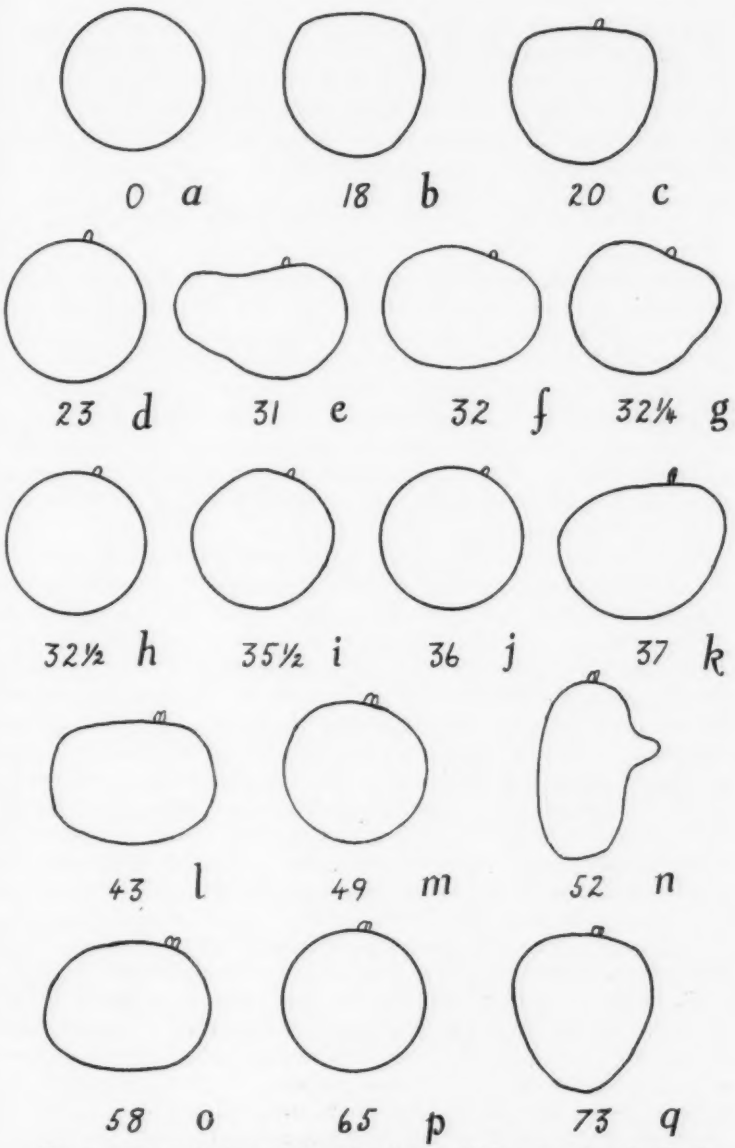

FIG. 3. Changes in shape of the nude egg of *Ascidiella aspersa* from fertilization to just before first cleavage. The figures indicate the time in minutes following insemination.

Since Dalcq accepted the site of the polar-pit as being identical with that of the polar-body and used it to orient the eggs for cutting, it follows that some doubt is thrown on the validity of his conclusions regarding the localization of presumptive germinal regions in the unfertilized egg of *Ascidiella aspersa*, although, of course, not on the existence of such presumptive regions.

Changes in Egg Shape from Fertilization to First Cleavage

Changes in the shape of an egg from the time of insemination to the first cleavage have been recorded. Recently Hoadley (1934) has given an account of such changes in the egg of *Nereis* and has related the movements to definite intervals from insemination to first cleavage, finding that the most marked changes in shape occur from the time of insemination to the breakdown of the germinal vesicle, but after being interrupted by "rest intervals" the changes continue to the time of first cleavage. Whereas the above observations were made on the egg of *Nereis* enclosed in its membrane, the observations recorded here were made on nude eggs.

The egg of *Ascidiella aspersa*, when removed from the oviduct, is enclosed by two membranes, separated by a space. The inner membrane adheres closely to the surface of the egg so that when development occurs with these membranes intact no changes in shape of the egg can be observed. However, if a nude egg be observed from the time of insemination to first cleavage, definite and marked changes in shape may be seen. The egg remains spherical until within about two minutes of polar-body formation, when it becomes flattened and somewhat elongated. This elongation is even more marked immediately following the extrusion of the polar-body, which occurs about twenty minutes after insemination (Fig. 2, *a–c*). About three minutes after the first polar-body is cut off, the egg returns to a spherical shape (Fig. 3, *d*) but this condition is not retained for long, the egg then undergoing a series of deformations. Figure 3, *e–i* shows the changes which occur from 31 to 35½ minutes after insemination. One half-minute later the egg has returned to a spherical condition (Fig. 3, *j*). A minute or two later the egg again loses its spherical shape, flattening at the animal pole, and the second polar body is cut off (Fig. 3, *k*). From the time of formation of the second polar-body to first cleavage the egg continues to undergo changes in shape, returning to a spherical or nearly spherical condition from time to time. Figure 3, *l–q* shows the changes in shape in the latter period at intervals of a few minutes.

The cause of these changes was not investigated experimentally. Unlike the egg of *Nereis*, the germinal vesicle of the egg of *Ascidiella aspersa* is ruptured upon entering the oviduct, hence before fertilization. Consequently the changes in egg shape cannot be referred to the rupture of the germinal vesicle. Nevertheless, it appears likely that the changes in shape are associated with a transitory decrease in viscosity of the protoplasm. At the same time the surface membrane of the egg must possess considerable elasticity—despite its great delicateness, as is evidenced by the facility with which it is ruptured upon handling—to be able to undergo such changes as are shown, for example, in Fig. 3, *n*.

History of Stain from First Polar Body to Tadpole

After the first polar body has been extruded and the egg has returned to a spherical condition, there is present a clear unstained cap of protoplasm at the animal pole and a thinner clear protoplasmic cap at the vegetal pole; the remainder of the egg appears to be homogeneously stained (Fig. 4, *a*). Some ten odd minutes before first cleavage the centre of the stained egg becomes less deeply stained and finally almost clear, and is the first indication that the nucleus is preparing to divide. This clear area extends across the egg at right angles to the plane of the first cleavage, and at the same time the beginning of cytoplasmic cleavage becomes evident (Fig. 4, *b–c*). During these changes which lead to the first cleavage it is not possible to identify definitely the clear vegetal cap, but with the formation of the two-cell stage it is seen that it has divided so that each of the two blastomeres possesses a clear cap of protoplasm at the vegetal pole (Fig. 4, *d*). The latter figure also shows that the stain has become largely restricted to the vegetal part of the egg, only extending towards the animal pole, but not completely covering it, as a thin pheripheral layer. After the second cleavage the clear cap of the vegetal pole can no longer be followed. Figure 4, *e* shows a four-cell stage from the animal pole; the stain appears to be distributed about the periphery of each of the blastomeres with the exception of a narrow neck of unstained protoplasm joining the clear central mass of protoplasm of the anterior and posterior blastomeres of each side. The stain is not as intense in the anterior blastomeres and in some specimens appears not to cover completely the animal portion of the anterior blastomeres. Probably the correct interpretation of the discrepancy in the distribution of the stain in the anterior blastomeres is that the stain is present in the vegetal part but not in the animal part.

In the eight-cell stage (Fig. 4, *f*) the four animal blastomeres are

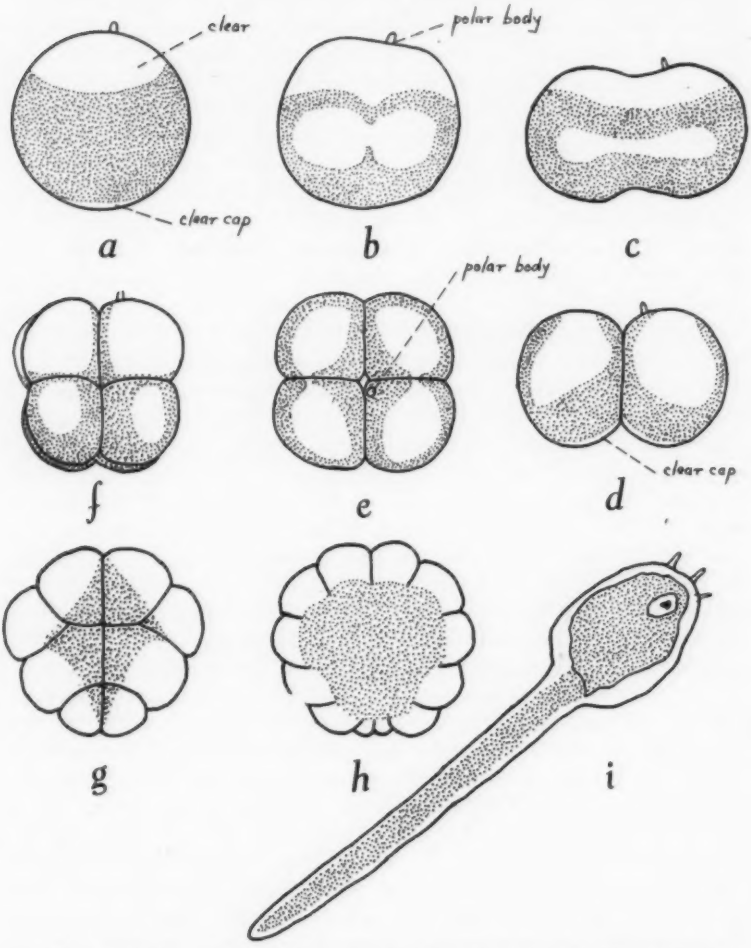

FIG. 4. Nude egg of *Ascidiella aspersa* vitally stained with Nile Blue. The dots indicate the distribution of the stain. (*a*) about three minutes after the first polar body has been extruded; (*b*)–(*c*) early stages of first cleavage; (*d*) two-cell stage; (*e*) four-cell stage from the animal pole; (*f*) eight-cell stage, (*g*) sixteen-cell stage, viewed as a transparent object; (*h*) approximately sixty-four-cell stage, viewed as a transparent object; (*i*) twenty-four-hour tadpole.

almost devoid of stain, which is mainly confined to the vegetal blastomeres. Figure 4, *g–h* shows a 16-cell stage and an approximately 64-cell stage respectively, viewed as transparent objects. It is evident that the stain has become almost entirely restricted to the future endodermal and chordal regions. This conclusion is borne out by the distribution of the stain in the 24-hour tadpole (Fig. 4, *i*), from which it will be seen that the stain is present in the endoderm (gut) and the notochord, the latter being somewhat less densely stained than the former.

Summary and Conclusions

1. The observations recorded are on the living nude eggs of *Ascidiella aspersa* and *Phallusia mammillata*, as obtained in the vicinity of Plymouth and stained with Nile Blue and Neutral Red.

2. In the unfertilized egg the periphery of the egg stains more intensely than the central region, the boundary between the two regions being usually well-defined. One minute area of the periphery remains clear and unstained—this is the so-called "polar pit."

3. The statement by Dalcq (1932) that the "polar pit" marks the site of the second polar body could not be confirmed. On the contrary, evidence is presented which strongly indicates that there is no relationship between the site of the "polar pit" and the site of extrusion of the polar body. This observation throws doubt on Dalcq's conclusions regarding the topography of presumptive germinal regions in the unfertilized egg, though, of course, not on their existence.

4. The changes in shape which the egg undergoes from the time of insemination to first cleavage are recorded. The changes start with a flattening of the egg about two minutes before the first polar body is extruded. About three minutes after the latter event the egg returns to its spherical shape. Shortly afterwards the egg undergoes a series of deformations which last until the first cleavage, being interspersed with periods during which the egg returns to a spherical condition. The changes in shape indicate that the extremely delicate egg membrane nevertheless possesses a high degree of elasticity.

5. Just before the first polar body is cut off the distribution of the stain changes. With the formation of the first polar body it is seen that there is present a cap of clear unstained protoplasm at the animal pole, and a thin cap of clear unstained protoplasm at the vegetal pole. In the young tadpole the stain is found to be confined to the endoderm (gut) and notochord. It seems probable that the movements of the stain indicate the changes which the presumptive chordo-endoplasm undergoes from the unfertilized egg to the time when it becomes definitive endoderm and notochord in the young tadpole.

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STUDIES OF THE MITOTIC FIGURE

V. The Time Schedule of Mitotic Changes in Developing Arbacia Eggs

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Purpose of the Study

In order that the physiological variations so frequently studied in developing *Arbacia* eggs may be related to the accompanying cytological changes, this paper reports the time schedule of the mitotic events from fertilization to third cleavage.

It is well known that the presence of pigment granules in the living eggs of *Arbacia punctulata* makes it difficult to determine the exact time when many of the cytological changes occur. The time schedule can be worked out completely only in fixed eggs. The facts thus obtained, however, can readily be applied to experiments with living eggs, because, in any given egg-set, as will be demonstrated later, there is a constant relation between the time when it divides and when each mitotic stage occurs. In other words, one can subject *Arbacia* eggs to experimental treatment at various intervals after fertilization, and, having determined the cleavage time, can by the use of such a time schedule calculate the mitotic stage which was present at the time of treatment.

The Mitotic Time Schedule of Arbacia Eggs

The egg-set of one female was employed in the present study, and eggs were fixed at close intervals with Bouin's reagent. The temperature was kept at 20° ± 0.2. The material was sectioned at a thickness of 5 μ, and stained with Heidenhain's hæmatoxylin. At each interval of fixation 100 eggs, chosen at random, were studied at a magnification of 600 ×, to determine the mitotic phases present.

The data are presented numerically in Table I, and in graph form in Chart 1. Photographs of living eggs are also shown, in Plate I.

In making a mitotic time schedule, the manner in which the various stages are defined is an important factor. For example, in the present study telophase (Fig. 8) is considered as beginning when the solid anaphase chromosomes have become numerous small vesicles,

and as ending when they have fused to form a single nucleus; the resting phase (Fig. 9) begins there and ends when the prophase chromosomes appear. During both phases the asters gradually fade; they are less clearly organized during telophase than during anaphase; and they become exceedingly vague during the resting condition, finally disappearing just as the new prophase asters arise. However, these two phases might also be defined in terms of changes in the asters, as well as in the nuclear materials, in which case both might be regarded as

TABLE I

The mitotic time schedule of Arbacia eggs from fertilization to third cleavage. The eggs of but one female were used. The temperature was 20° C. ± 0.2. One hundred eggs are reported at each interval of fixation (Bouin's fluid); hence the number of eggs in each phase is its percentage of the total. The figures referred to are shown in Chart 1.

Pronuclei and sperm-aster

Minutes after fertilization	2½	5	8½	11	13	16	20	25			
Nuclei not touching (Fig. 1)	97	92	79	47	8	4					
Nuclei touching (Fig. 2)	3	8	21	53	90	60	19	3			
Nuclei fused (Figs. 3 and 4)					2	36	81	97			

First-cleavage figure

Minutes after fertilization	30	35	40	45	50	55	60	65	70	75	80
Nuclei fused (continued)	98	93	90	60	14	4					
Prophase, early (Fig. 5)	2	7	10	40	40	20					
Prophase, late					46	37	8	6			
Metaphase (Fig. 6)						39	55	7			
Anaphase, early							34	20			
Anaphase, late (Fig. 7)							3	32	6		
Telophase, early								35	20		
Telophase, late (Fig. 8)									66	6	4
Resting (Fig. 9)									8	94	90
Next prophase, early											6

Second-cleavage figure

Minutes after fertilization	85	90	95	100	105	110	115	120	125		
Resting (continued)	62	30									
Prophase, early (Fig. 10)	36	58	35	8							
Prophase, late	2	12	43	30	5						
Metaphase (Fig. 11)			22	45	25						
Anaphase, early				11	22	4					
Anaphase, late (Fig. 12)				6	26	18	4				
Telophase, early					13	18	12	2			
Telophase, late (Fig. 13)					7	42	30	10	6		
Resting (Fig. 14)					2	18	54	84	76		
Next prophase, early								4	18		

TABLE I (cont'd)

Third-cleavage figure

Minutes after fertilization	130	135	140	145	150
Resting (continued)	18	2			
Prophase, early (Fig. 15)	54	46	7		
Prophase, late	22	26	18	4	
Metaphase (Fig. 16)	6	19	51	11	3
Anaphase, early		6	14	12	5
Anaphase, late (Fig. 17)		1	10	38	12
Telophase, early				21	29
Telophase, late (Fig. 18)				11	18
Resting				3	31
Next prophase, early					2

First Cleavage	Minutes after fertilization	67	68	69	70	71	72	
	Percentage of cleaved eggs	20	29	58	69	76	85	
Second Cleavage	Minutes after fertilization	104	105	106	107	108	109	110
	Percentage of cleaved eggs	10	23	34	54	64	66	76
Third Cleavage	Minutes after fertilization	139	141	143	145	146	148	150
	Percentage of cleaved eggs	3	10	34	39	66	81	88

different periods of telophase, the time when the asters are disappearing. In that event the resting phase would be regarded as late telophase, and the shape of the telophase curve would differ from that here reported.

There are difficulties in applying any given set of definitions of mitotic phases to the division figures in various cell types, because asters may be present or absent, the resting period may be long or practically non-existent, and there may be other variations. For this reason the definitions of the mitotic phases used in the present work are given here.

The history of the pronuclei and the sperm-aster falls into three periods: first, when the pronuclei are not yet in contact and the aster is small, delicate, and round; second, when the pronuclei are touching but have not yet fused, and the aster is medium in size but more distinct; and third, when the pronuclei have fused but the prophase configuration has not yet appeared. This is a long period, during which the identity of the male pronucleus is gradually lost in the female pronucleus, and the large round aster becomes sharply elongate and then slowly disintegrates. In *Arbacia* eggs the history of the sperm-aster is atypical in two respects: first, during the late history it is crescent-shaped instead of round; and second, this stage persists an unusually long time. On the other hand, the first-cleavage figure of *Arbacia* eggs is typical of those of most invertebrate eggs.

Prophase, early, begins when the chromosomes first appear as vaguely-defined, thread-like structures which stain darkly; the nuclear membrane is still intact. At this time two new small asters arise. The period ends when the nuclear membrane dissolves.

Prophase, late, begins with the dissolution of the nuclear membrane. The well-formed chromosomes are irregularly scattered on the spindle, but have not yet become aligned in the metaphase plate. During this time the asters increase in size and distinctness.

Metaphase covers only the period when the chromosomes are definitely aligned in the metaphase plate. During this time the asters enlarge slightly and become somewhat more distinct.

Anaphase, early, begins with the separation of the metaphase chromosomes and ends when they have moved about halfway toward the spindle-ends.

Anaphase, late, begins with the latter condition, and ends when the chromosomes have reached the spindle-ends, still in the form of typical densely staining rods. During anaphase the asters attain their maximum size and distinctness.

Telophase, early, begins with the vacuolization of the chromosomes, accompanied by their reduced capacity to stain with Heidenhain's hæmatoxylin. It ends when the fusion of the vacuoles has proceeded about halfway.

Telophase, late, includes the period of the final fusion of the vesicles, and ends when the single rounded nucleus is fully formed. During telophase the aster begins to disintegrate; the fixed rays are more irregular in shape and less distinct than those of anaphase.

The resting stage covers the period when the nuclear materials have the form of a typical nucleus; none of the substances stain with Heidenhain's hæmatoxylin. During this time the asters continue to fade; they disappear about the time when the next prophase asters arise.

The method of defining mitotic phases is not the only cause of discrepancy in making mitotic time schedules. If counts are made only at five-minute intervals, the exact point when a given phase occurs in greatest numbers may not be ascertained; again, it is difficult to decide to which phase to assign an egg in a transitional stage between two phases; furthermore, if not more than 100 cells are counted at each interval, a certain error is involved.

Variation in the Mitotic Time Schedule of Arbacia Eggs

The variability in behavior among the egg-sets of different *Arbacia* females is well known. ". . . freshly shed eggs, from freshly collected individuals, vary widely in a number of morphologic and physiologic traits, such as size, color, shape of eggs, amount of jelly, duration of agglutination, rate and manner of membrane formation, rate and percentage of segmentation, etc." (Goldforb, 1929, p. 350.)

The time of cleavage of the egg-sets of 88 females is presented in Table II. As these data show, there is a variation of about 10 per cent in the cleavage time of different egg-sets obtained from females collected at the same time and place, fertilized simultaneously, and allowed to develop under identical laboratory conditions.

Seasonal variation is also apparent. The egg-sets of the 61 females studied during August have an average cleavage time of 46.6 minutes, while that of the 27 egg-sets studied in September is 50.4 minutes—a fluctuation of about 8 per cent.

The effects of changes in temperature upon time of cleavage need no comment. Under ordinary conditions at the Marine Biological Laboratory, the temperature varies from about 18° to 25° C. during the *Arbacia* season. Cleavage time within this range varies from 40 to 68 minutes (Loeb and Wasteneys, 1911)—a difference of more than 50 per cent. Harvey (1932, pp. 149–150) has summarized the results of other studies of temperature effects.

The Applicability of the Present Data to Studies of Arbacia Eggs Generally

Since *Arbacia* eggs vary so greatly in cleavage time under various conditions, the usefulness of any time schedule of mitotic events will depend upon whether or not there is any constant relationship between the time when each mitotic phase occurs and the cleavage time.

That there is such a constant relationship is shown by the data of Table III, which presents the mitotic time schedules of the first-cleavage figure in five egg-sets, in which the experimental conditions differ as to year, time of season (July 6 to September 20), and temperature (from 15° to 25° C.). Regardless of the length of the cleavage cycle in these sets, the time when each mitotic phase occurs is the same percentage of the cleavage time. The minor deviations are random in nature and due to experimental errors.

Not only do the various mitotic phases of the first-cleavage figure in the present study occur at the same, or practically the same, percentages of cleavage time as the four other studies reported in Table III, but in the case of three of those studies the data available (but not reported) show a similar constant relationship between the phases of the sperm-aster and of the second-cleavage figure. Hence the data of the present study can be applied to *Arbacia* eggs generally. The various mitotic phases occur at the following percentages of cleavage time:

Pronuclei and Sperm-aster

Early (Fig. 1)	7 per cent of cleavage time
Middle (Fig. 2)	17 per cent
Late (Figs. 3 and 4)	43 per cent

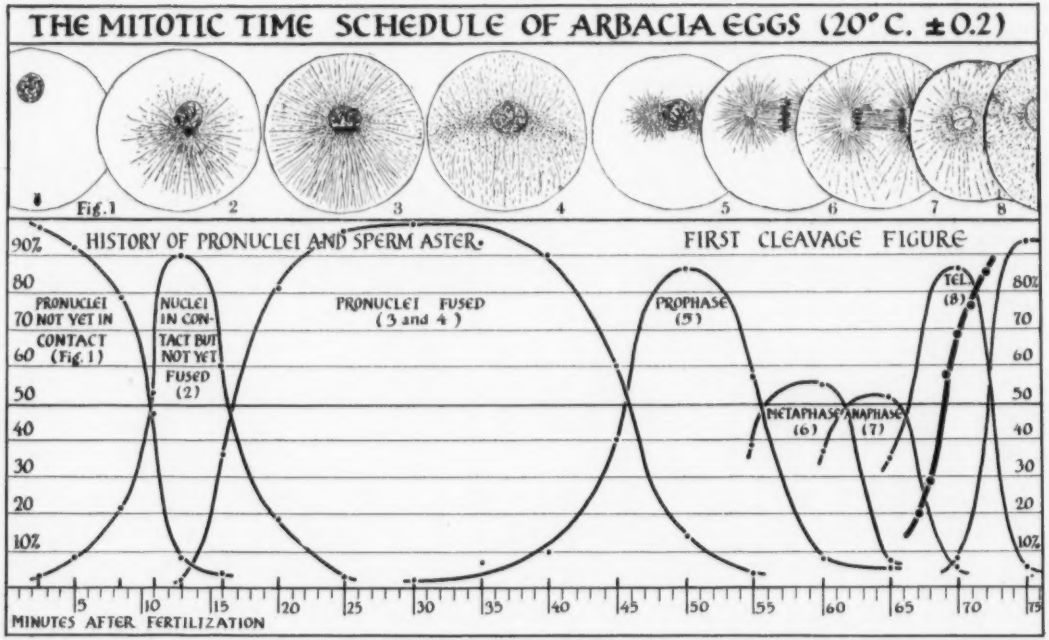
First-Cleavage Figure

Prophase (Fig. 5)	72 per cent
Metaphase (Fig. 6)	85 per cent
Anaphase (Fig. 7)	93 per cent
Fifty per cent first cleavage	100 per cent
Telophase (Fig. 8)	101 per cent
Resting (Fig. 9)	112 per cent

Second-Cleavage Figure

Prophase (Fig. 10)	136 per cent
Metaphase (Fig. 11)	147 per cent
Anaphase (Fig. 12)	152 per cent
Fifty per cent second cleavage	155 per cent
Telophase (Fig. 13)	161 per cent

CHART 1


THE MITOTIC TIME SCHEDULE OF ARBACIA EGGS (20° C. ± 0.2)
Fig. 1
2
3
4
5
6
7
8
HISTORY OF PRONUCLEI AND SPERM ASTER.
FIRST CLEAVAGE FIGURE
90%
80
70
60
50
40
30
20
10%
PRONUCLEI NOT YET IN CONTACT (Fig. 1)
NUCLEI IN CONTACT BUT NOT YET FUSED (2)
PRONUCLEI FUSED (3 and 4)
PROPHASE (5)
METAPHASE (6)
ANAPHASE (7)
TEL. (8)
5
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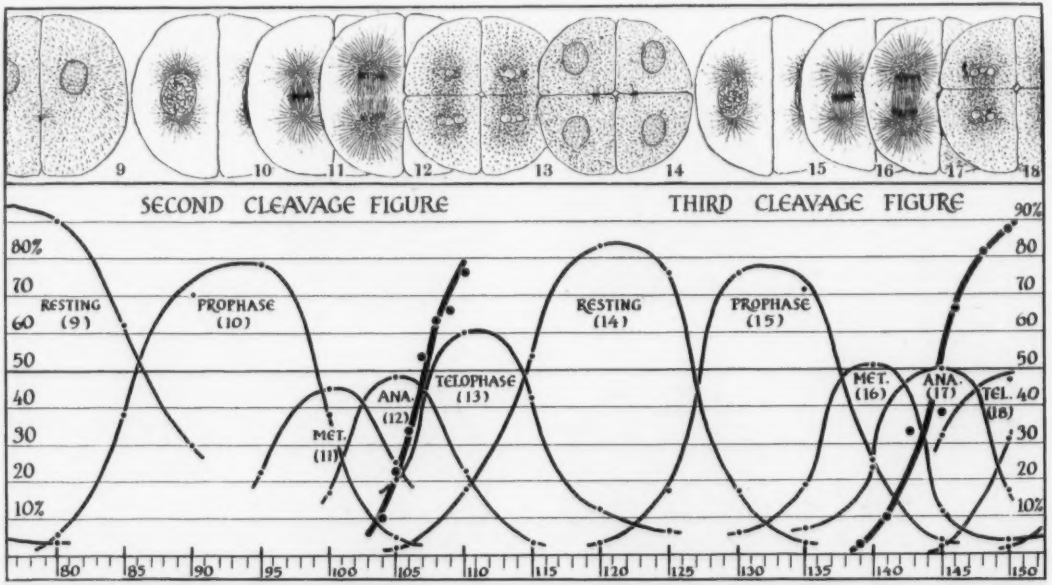
75
MINUTES AFTER FERTILIZATION



This chart presents the data of Table I in graph form. The ordinate shows the percentage of the various mitotic phases at each of the intervals reported, the latter shown on the abscissa. The three heavy lines show times of cleavages. The drawings were made in the same manner as those in a previous study (Fry and Parks, 1934, p. 477); they are enlarged 500 ×. Bouin's reagent was used, which causes a shrinkage of about one-third. The third-cleavage figures lie in a plane at right angles to that of the preceding ones, but this is not shown in the chart.

Methods of Securing Arbacia Eggs Which Have the Same Mitotic Time Schedule

In many studies of *Arbacia* eggs variations in the cytological time schedule from one egg-set to another are of no significance. But where modifications in physiological factors are to be related to morphological changes, it is important to know the times of such changes as accurately as possible. If the egg-set of a single female is insufficient for an experiment, it is desirable that the several sets used have similar time schedules. Furthermore, it may be advisable that the eggs used in successive daily experiments are similar in this respect.

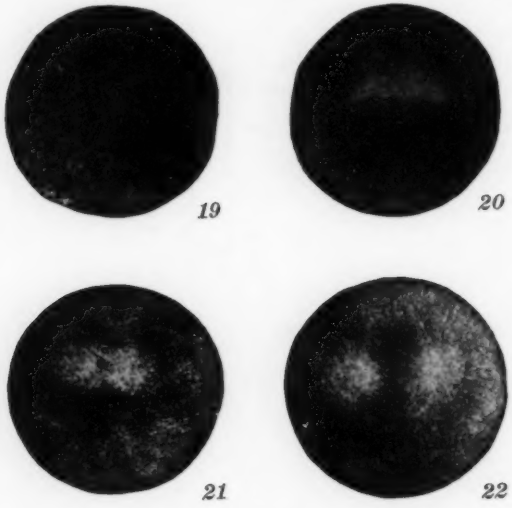


Plate I

Photographs of the major mitotic configurations seen in living Arbacia eggs from fertilization to first cleavage.

The prints are from moving picture films made by the writer (Figs. 19 and 20) and by Professor Robert Chambers (Figs. 21 and 22), both with the aid of Mr. C. G. Grand. Figures 21 and 22 were taken under slight compression, which makes the structure more visible than it usually is. The photographs were made at a magnification of 250 ×, and are here reproduced at 460 ×.

Fig.	Mitotic stage	The same stage after fixation (Chart 1)
19	Pronuclei in contact or just fused; sperm-aster round	Figs. 2 and 3
20	Pronuclei fully fused; sperm-aster elongate	Fig. 4
21	Prophase or metaphase of the first-cleavage figure	Figs. 5 and 6
22	Anaphase or telophase of the first-cleavage figure	Figs. 7 and 8

TABLE II

Variation in cleavage time among egg-sets of different females. All sets reported were in optimum condition, as shown by practically 100 per cent regular cleavage. The temperature was 23° ± 0.5. (For method of determining cleavage time see p. 98.) *Result:* On any one day there is a variation of about 10 per cent in the cleavage time of different egg-sets; between egg-sets obtained in August and in September there is an average difference of about 8 per cent.

Date 1934	Distribution of egg-sets with reference to cleavage time (i.e., time from fertilization until 50 per cent of eggs have cleaved)										
	44′	45′	46′	47′	48′	49′	50′	51′	52′	53′	Average
Aug. 4		1	1	1	1						46.5
Aug. 6			2								46.0
Aug. 7					3		1				48.5
Aug. 8	3	4	2	1	1						45.5
Aug. 10	1	1	2	1	2	1					46.6
Aug. 11	1	2	3								45.6
Aug. 12					2	1					49.0
Aug. 18	1	1	2	1	2	1					46.6
Aug. 20	2	1		1	1						45.6
Aug. 22		1	4	2	2	1					46.8
Sept. 14						2	2	3	2	2	51.0
Sept. 15					1	1	1	3	2	2	51.0
Sept. 20					2	2	1		1		49.3

TABLE III

The mitotic time schedule of Arbacia eggs during the first-cleavage cycle under various laboratory conditions. The data of egg-sets I, II, and V are from a study of the effects of temperature upon mitosis (now in progress); set III is the material of the present study; set IV is the material used in a study of viscosity and mitosis (Fry and Parks, 1934). *Result:* In spite of differences in temperature and season, and variations among egg-sets of different females, the mitotic phases always occur at the same proportional part of the cleavage time. The slight deviations are due to experimental errors.

Experimental conditions			Time schedule									
			Prophase		Metaphase		Anaphase		Cleavage		Telophase	
Egg-set	Date	Temp.	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time
		°C.										
I	Aug. 4, 1934	25	30	72	35	83	38	90	42	100	43	102
II	Aug. 12, 1934	20	50	74	56	85	63	94	67	100	68	101
III	Sept. 20, 1934	20	50	72	59	85	64	93	69	100	70	101
IV	July 6, 1932	20	47	71	57	86	63	95	66	100	69	104
V	Aug. 6, 1934	15	75	67	97	86	105	93	113	100	117	103

Obviously the mixing of egg-sets with different time schedules causes a "spread" in the time when any given mitotic stage occurs.

The term *cleavage time* is used here to include the period from fertilization until 50 per cent of the eggs have divided. To determine when any one egg has divided is not a simple matter. A constriction appears first on one side; about a minute and a half later, at 24° C., it is seen on the opposite side; complete division requires another minute. In making the counts of the present study, eggs having a constriction on but one side were arbitrarily counted as uncleaved, and those having constrictions on both sides as cleaved.

In order to secure different egg-sets [1] which have the same cleavage time, preliminary tests are required. The egg-set of each female is kept separate; samples of each are fertilized simultaneously, and kept at the same temperature. When the time of cleavage approaches, the samples are observed in rapid rotation. Since one observation requires about 15 seconds, it is not advisable to work with more than eight or ten samples at a time. If larger numbers of females must be tested, samples are fertilized in groups at about 15-minute intervals. When observing cleavage, accuracy is increased if the eggs are gently stirred with a glass needle, so that they can be seen from several positions. The sets which have the same cleavage-time are then mixed and used for the experiment.[2]

When such preliminary tests are made, the eggs to be used later in the experiment are kept standing for somewhat over an hour. This, however, does not modify either the percentage of normal cleavage or the time when it occurs. Several experiments were carried out to determine the effects of such standing upon the eggs, and it was found that cleavage is unmodified for at least six hours.

This preliminary test is especially valuable when working with *Arbacia* eggs late in the season. Those familiar with this material rightfully distrust experiments carried out toward the end of the season, when the eggs of many females are in poor condition. Since it is the usual practice to mix the egg-sets of a number of females, the inclusion of subnormal sets introduces doubtful factors into the experiment. By preliminary tests, however, one can select the sets which

[1] Methods for obtaining eggs and fertilizing them have been described by Just (1928). Additional details will be found in the writer's discussion of *Arbacia* eggs in the forthcoming *Compendium of Culture Methods for Invertebrate Animals.*

[2] The procedure described is sufficiently accurate for most purposes; if greater accuracy is necessary, the following method is generally employed. In any given sample, as soon as a small percentage of the eggs have cleaved, some of them are fixed in a 0.5 per cent solution of formol in sea-water; this is repeated at intervals until the majority have divided. Eggs thus fixed have practically the same appearance as when alive, and accurate counts can then be made.

are in optimum condition, as judged by capacity to lift distinct fertilization membranes, to cleave regularly in practically 100 per cent of the cases, and to develop into top-swimming plutei.

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THE EFFECT OF WHITE LIGHT UPON THE RATE OF DEVELOPMENT OF THE RHIZOID PROTUBERANCE AND THE FIRST CELL DIVISION IN FUCUS FURCATUS[1]

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It has been observed by several investigators that the site of germination and the plane of the first cell division in the egg of the brown alga, *Fucus*, are determined by the direction of a beam of light. The rhizoid develops on the side of the egg which is least illuminated, provided, as Hurd (1920) and Kniep (1907) and others have observed, the individual eggs are at least several egg diameters apart. If egg cells lie more closely together they exert certain mutual influences upon the developmental axes which overcome the light effect. Hurd (1920) also found that the most effective part of the visible spectrum is in the blue range.

The present paper gives the results of experiments designed to measure the effect of light upon the rate of the early developmental changes in *Fucus* when eggs are spaced far enough apart to avoid mutual effects, so that the axes of differentiation are determined by the direction of illumination. Since these experiments were carried out, a search of the literature has shown that Kniep (1907) long ago noted in passing a slight accelerating effect of light on the germination of *F. vesiculosus* and *F. serratus* from the Norwegian Coast. His observation is confirmed and extended in this investigation on *F. furcatus*. A considerably more marked effect is also found.

Material

In the year 1934–35 *Fucus* has been collected at least once a week from early October until the middle of June at Moss Beach, about twenty miles south of the Golden Gate on the California coast. Two similar but distinct types of *Fucus* were found here in fair abundance, in slightly different habitats. On a large tidal shelf, a shorter type has narrower fronds and receptacles, while on a reef a larger more robust type which grows to greater length has relatively wider fronds and receptacles. *Fucus* is apparently plastic in regard to growth forms. To avoid if possible the question of growth forms and genetic forms,

[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

collection has been confined to what is believed to be one uniform type (the longer wider type) taken from the reef. A typical sample, collected early in June, is shown in Fig. 1. From Gardner's (1922) descriptions and classification, this could not be other than *Fucus furcatus*. Professor N. H. Gardner has been so kind as to identify living samples of the material which was used as *F. furcatus* f. *luxurians*, and of the smaller narrower type which was distinguished and avoided as *F. furcatus* f. *typicus*.

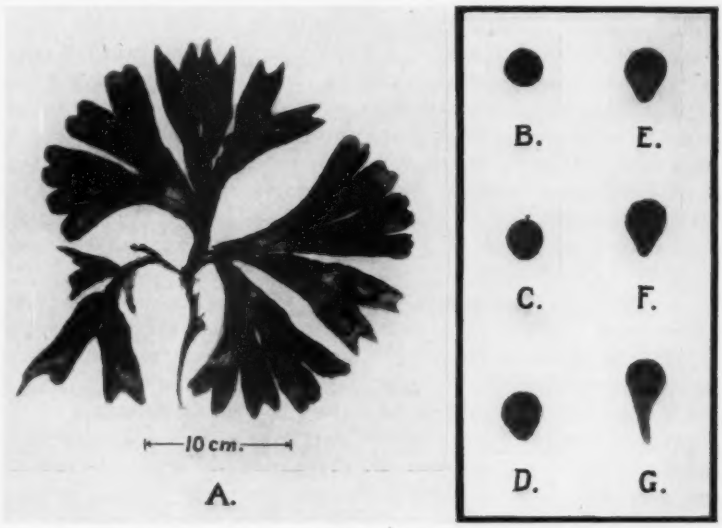

FIG. 1. (*A*) Photograph of a typical sample of the type of *Fucus* used, which has been identified as *F. furcatus* f. *luxurians* (collected early in June). The scale applies only to (*A*). (*B*)–(*G*) Developmental stages showing the development of the rhizoid protuberance and cell and (*G*) a later stage. A large egg of this form is approximately 90 microns in diameter.

Throughout the period referred to, mature gametes in excellent condition have been obtained. In the winter months new fruiting tips began to grow, and these fruited in late March, yielding larger numbers of gametes than the older tips, which, however, did not cease to bear if at all at least until well after the new tips were ripe. The new tips continued steadily to come more fully into fruit throughout the spring until and including the middle of June when collection was discontinued at Moss Beach. The present experiments were carried out on material collected in January and February.

METHOD

After some experimentation, an effective standard procedure was devised for caring for the material. Fruiting tips were cut off with uncontaminated scissors and placed in a gallon thermos jug which had been pre-cooled with ice. A corked bottle of ice was put in on the tips and the jug was sealed. The tips were thus kept cold and moist, but not in sea water. The jug was kept in an electric refrigerator at 4° C. as the working source of supply. Sea water was collected at the same time and after being twice passed through coarse filter paper was stored at 8° C. Fresh gametes were obtained by first washing a number of tips thoroughly in 8° C. sea water, to remove debris and previously shed gametes, and then placing them in a dish of sea water at 15° C. This served as a stimulus to shedding, and in about twenty minutes in the spring (40 minutes in the fall) eggs and sperm capsules were shed. After the tips had been in the refrigerator for three days, large numbers of gametes were obtainable, while before this time often few or none would exude. The material is good at least for well over a week when kept at 4° C., although none was used which was more than a week old.

This form is hermaphroditic, as are all the Fucaceæ on the California coast. When it was necessary to know the approximate time of fertilization, as in the present instance, a group of recently shed capsules were transferred to another dish of sea water at 8° C., where the time at which 50 per cent of the egg-bearing capsules had broken down was noted.[2] Eight eggs occur in a capsule which dissolves in sea water and ruptures, when fertilization ensues if antherozoids are present. Very small capsules containing many antherozoids are shed along with the egg capsules and many of these rupture before the egg capsules do so that motile antherozoids, or sperm, are ordinarily abundant about and on the egg capsules when these break down. In the case of an isolated capsule the time of fertilization of the eight eggs can be determined quite precisely. In the populations of capsules of nearly equivalent age in sea water, the time between rupture of the first and last capsule was usually about 15 minutes, so that the midpoint represented the time of fertilization of all of the eggs within approximately ± 8 minutes.

After capsule breakdown, the eggs were kept in a dark constant temperature room in which the air temperature was 14¾ ± ¼° C.

[2] It was not entirely necessary to have the capsules break down at 8° C. instead of at 15° C., but uniformly single eggs emerge at cool temperatures, while as the temperature increases, so does the proportion of eggs which fuse to form polyploid giants (Whitaker, 1931).

The heat capacity of the water about the eggs reduced the temperature variation considerably. The eggs were in the dark except for brief exposures to dim red light, or during experimental illumination.

Following fertilization, the *Fucus* egg secretes a gelatinous substance which gradually becomes sticky and finally hardens to form a cellulose wall (Whitaker, 1931) which attaches the egg quite firmly to such substrates as glass or agar jel. In manipulating *F. furcatus* eggs at 15° C., the stickiness becomes conspicuous about 45 minutes after fertilization, and is very pronounced at 2 hours. The manipulation, transfer, and placing of eggs was completed in these experiments within 20 minutes after fertilization.

A large double compartmented box was constructed inside the constant temperature room, painted black inside and out. It was so arranged that a light bulb, in fixed position at one meter distance, laterally illuminated a levelled platform in one compartment, while a similar platform in the other compartment remained in complete darkness. An electric fan was so arranged, in conjunction with baffles and slits which admitted no light, that copious and equal volumes of air passed continually from the constant temperature room through the experimental chambers and out behind the light. Calibrations under working conditions showed that the two chambers were at the same temperature, and at the same temperature as the constant temperature room, namely $14\frac{3}{4} \pm \frac{1}{4}$° C. The white light source used in the experiments was a General Electric frosted 40-watt bulb, operated on 110 volts, A.C. With facilities and assistance kindly provided by Dr. Arthur Giese, this was calibrated by means of a thermopyle against a standard light. The intensity under working conditions at one meter distance from the bulb was such as to give total energy of approximately 1500 ergs per second per cm.^{2}.

Measuring Time Lapse

Soon after fertilization, eggs were thinly scattered in sea water over the bottoms of square Petri dishes (8.5 cm.^{2}) which presented flat surfaces to the laterally transmitted light, and also served as moist chambers. In any given experiment, the eggs reared in the light and in the dark were arranged by equipartition and random dispersion to be of equal concentration and statistical proximity on the bottom of the dish, as well as to be of equal concentration per unit volume of sea water. In the different experiments the same conditions in these regards were approximated. The average concentration of eggs was of the order of 25 eggs per cm.^{2} of bottom, and 60 eggs per cc. sea water. The eggs are about 65–90 microns in diameter. In recording results

all eggs were ignored which were within 5 egg diameters of another egg, because of certain mutual effects of these eggs when they lie within two or three egg diameters of neighbors. The great majority of the eggs were much more than 5 egg diameters apart.

In Experiments 9 and 10 (Fig. 2) duplicates were run in isosmotic 1 per cent, and in Experiment 9 also in 2 per cent, dextrose. Solutions of dextrose in triple distilled water were made approximately isosmotic with sea water (11/12 M). Enough of this was added to sea water to give a resulting solution containing one, or in the 2 per cent two, grams of dextrose per 100 cc.

Within 20 minutes after fertilization one set of dishes bearing eggs was placed in the illuminated chamber of the dark box, and another set in the dark chamber. After preliminary survey, two arbitrarily chosen criteria of developmental stage were selected which could be quite sharply identified. The first of these was the early but unmistakable protuberance at one side of the egg, which is to give rise by extension and cell division to the rhizoid cell. The first cell division in *Fucus* results in two morphologically different cells which ordinarily lie side by side in the plane of the substrate (Fig. 1) and which by further division give rise respectively to the thallus and the rhizoid of the new plant. The developmental axis is thus, in the normal course, determined by the time the rhizoid protuberance appears, and is indicated by it. The second end point selected was the development of the cell plate to an arbitrarily chosen stage which was distinctly and sharply distinguishable, and which marked the beginning of the 2-cell stage. In the absence of cytological study, it is not certain precisely to what extent the cell plate had divided at this time.

The chief measure sought was the time after fertilization, in the dark and in the light, when 50 per cent of the population had reached (or passed) the stages in question. In the first seven experiments more numerous counts were made principally just before, during, and just after this time had been reached. It was then found to be both more convenient and more accurate, as well as to involve less disturbance of the dishes, to make percentage counts at less frequent intervals and to interpolate the 50 per cent point on the sigmoid curve which could be drawn through the points. This method was followed in Experiments 8, 9 and 10, the results of which are shown in Fig. 2. The eggs reared in the dark were observed and tabulated with red light, the eggs reared in the light with white light. Checks showed that the different lights used made no difference in identifying the stages. The Petri dish was moved back and forth across the stage of the microscope so that eggs were observed (with low power) as one might read a printed page. Each count involved from 100 to 300 eggs.

Results and Discussion

As may be seen in Table I, the time lapse from fertilization until 50 per cent of the population had attained (or passed) the two stages in question varied somewhat in the different experiments, but in all cases the rate of development was decidedly more rapid in the light than in the dark. Various factors which may affect the rate were naturally more uniform among the experimental groups of a given experiment than among different experiments. Therefore the ratio of the time lapse in the light and in the dark in a given experiment is a more useful measure for comparison, and such ratios are indicated at the right in Table I.

Table I

Time in hours from fertilization until 50 per cent of a thinly spaced population of eggs at 14¾ ± ¼° C. attained or passed the early rhizoid protuberance and the early cell plate stages.

Experiment number	Number of eggs	Time in hours after fertilization				$\frac{\text{Time in dark}}{\text{Time in light}} \times 100$	
		Fifty per cent rhizoid protuberance		Fifty per cent cell plate		Rhizoid protuberance	Cell plate
		Light	Dark	Light	Dark		
1 to 7	207 av.	15.2	20.0	22.2	25.5	132	115
8	182	12.7	17.0	20.5	24.0	134	117
9	146	13.7	17.7	22.1	25.1	130	114
10	145	14.4	16.5	22.9	25.3	115	110
Average.....		14.0	17.8	21.9	25.0	128	114

In deriving the final averages, Experiments 8, 9, and 10 as a group are weighted equally against Experiments 1–7 inclusive to arrive at what is probably the best approximation, since the former were carried out with special care and after more experience. The results of Experiments 8, 9, and 10 are shown graphically in Fig. 2, which also shows the time span of protuberance and cell plate formation in a population. It is seen (Table I) that it takes about 28 per cent more time for rhizoid protuberances to develop in the dark than in moderate white light (1500 ergs/sec./cm.^{2}), and about 14 per cent longer for cell plates to form. In absolute time, the rhizoid protuberance develops 3.8 hours sooner in the light, while the cell plate develops 3.1 hours sooner. The primary and actual acceleration in the light is therefore only in the processes leading to protuberance formation. The cell plate formation in fact loses some of the gain.

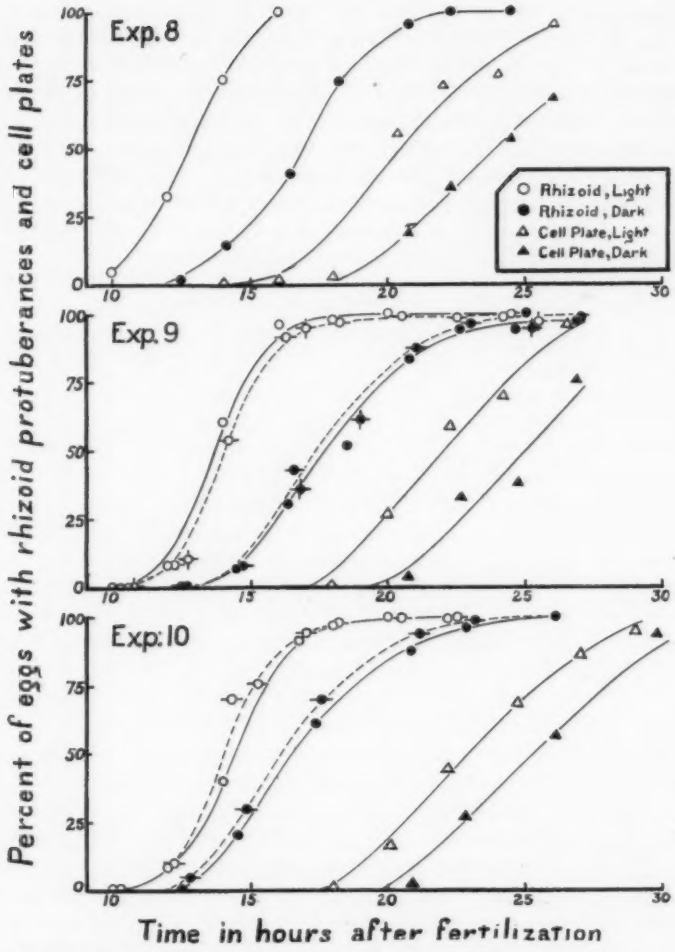

FIG. 2. Percentages of eggs in thinly spaced populations at 14¾ ± ¼° C. which attained or passed the stages indicated with passage of time after fertilization. In Experiments 9 and 10 the rhizoid protuberance points which bear two wings and four wings, through which dashed curves have been drawn, represent runs in sea water containing 1 per cent and 2 per cent dextrose respectively.

In Experiments 9 and 10 duplicate runs both in light and dark were made which differed from the main runs only in that the sea water contained 1 per cent dextrose (isosmotic), and in Experiment 9 also 2 per cent dextrose. The time lapses to the development of rhizoid protuberances are shown in Fig. 2. These results confirm the indications of rough preliminary tests in showing that the presence of 1 per cent dextrose in the medium does not speed up the development of the rhizoid protuberance in eggs in the dark to, nor appreciably toward, the rate in eggs in the light. A slight stimulation of the eggs in the dark compared with the eggs in the light is indicated (average = 2 per cent) but this is within the limits of error. Since it is not certainly known whether dextrose can enter these eggs, nor whether it must enter to be metabolized, no positive conclusion can be drawn from the negative effect of 1 per cent dextrose. As far as these results go, however, they suggest or at least leave the way open for the view that the stimulative effect of light upon the rate of formation of the rhizoid protuberance is not primarily a nutritive phenomenon.

Summary and Conclusions

1. Data are given upon the extended breeding season of the hermaphroditic *Fucus furcatus*, and means of obtaining uniformly fertilized unfused eggs are described.

2. When thinly spaced eggs are reared in filtered sea water at $14\frac{3}{4} \pm \frac{1}{4}°$ C., with lateral illumination by moderate white light (1500 ergs/sec./cm.^{2}), the time lapse from fertilization until 50 per cent of a population have developed early rhizoid protuberances (when the developmental axis is determined and indicated) is approximately 14.0 hours.

3. It takes approximately 28 per cent more time for the same development in the dark (at 17.8 hours).

4. Under the same conditions as in 2, 50 per cent of a population have developed cell plates, and have entered the early 2-cell stage, at approximately 21.9 hours after fertilization.

5. It takes approximately 14 per cent more time for the same development in the dark (at 25.0 hours).

6. White light thus markedly speeds up processes leading to the development of both of these structures. It accelerates the protuberance formation (involving softening of the cellulose wall on the side of the cell least illuminated) to a relatively greater extent. In absolute time, the protuberance formation is advanced 3.8 hours in the light, the cell plate 3.1 hours. The primary and actual acceleration is therefore only in the formation of the rhizoid protuberance, the cell plate formation merely holding most of the gain.

7. The presence of 1 per cent (isosmotic) dextrose in the sea water does not appreciably affect the rate of protuberance formation either in the dark or in the light.

The author is indebted to Mr. Edward Lowrance for assistance in carrying out the experiments.

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EXPERIMENTS ON LIGIA IN BERMUDA

IV. The Effects of Heavy Water and Temperature [1]

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Three previous papers of this series (Barnes, 1932, 1934, 1935) have described salt effects and space orientation of the littoral isopod *Ligia baudiniana* in Bermuda. Since this species of *Ligia* is a sub-tropical form living under fairly uniform temperature conditions, it was decided to establish the upper temperature range in air and in sea water and to determine the effect of immersion in concentrated heavy water, which is equivalent to a drop in temperature from the standpoint of energy content (*cf.* Eyring and Sherman, 1933; Barnes and Gaw, 1935; Barnes and Warren, 1935).

Methods

For the study of lethal temperatures a freshly collected adult was placed in a bottle of approximately 300 cc. capacity immersed in the water of a thermostat warmed by a knife heater controlled by a mercury relay. Fifty cc. of sea water or filter paper moistened with sea water were placed in the bottle. Air was pumped continuously through a spiral of glass tubing in the thermostat to bring it to the required temperature before it passed through the bottle. The time of death was taken as the point when the animal ceased all motion and would not respond when the bottle was violently jarred. All specimens were returned to sea water at normal temperature (27° C.), but none recovered after this end point was reached. Although the thermostat was accurate to 0.1° C., the temperature of the specimen bottle dropped 0.5 – 1° C. for a few minutes after the specimen was introduced.

For the determination of the temperature characteristics of the gill-beat, the animal was placed in 2 cc. of sea water in a test tube containing the bulb of a thermometer and a tube through which air was bubbled continuously. The test tube was immersed in a beaker of water at 27° C. and pieces of cracked ice were added with forceps to lower the temperature. Since only 2 cc. of 96 per cent heavy water were available, the experiment had to be carried out on a small scale

[1] Contributions from the Bermuda Biological Station for Research.

(including the controls). The temperature was constant to within 0.4° C. during one reading (time for 10 beats of the pleopods).

Further Experiments with Salt Solutions

Sea water was used as the immersion medium in the temperature studies although animals will not live indefinitely in this medium. Artificial sea water in which the two most toxic ions K and Mg were reduced did not give better results than natural sea water (Table I). It will be seen from the table, however, that artificial sea water with half the normal Mg content is more favorable than artificial sea water with the normal amount of Mg. As was pointed out in a previous paper (Barnes, 1935), the K content of artificial sea water has to be increased four-fold to produce easily recognized toxic effects. Three seasons work with *Ligia* has shown the great variation that occurs in

Table I

Longevity of Ligia in salt mixtures

Experiment No.	Solution *	Average longevity	Coefficients of variation	Maximum longevity	No. specimens tested
		hours		*hours*	
1	100 NaCl, 2.5 $CaCl_2$, 1 KCl, 11.6 $MgSO_4$	23± 2.3	10	96	36
	sea water control	61± 9.0	14	214.5	36
2	100 NaCl, 2.5 $CaCl_2$, 1 KCl, 5.8 $MgSO_4$	40± 4.0	10.3	288	63
	sea water control	52± 6.4	12.2	336	63
3	100 NaCl, 2.5 $CaCl_2$, 1 KCl	20± 2.5	12.4	89.5	32
	sea water control	82±12.4	15	288	32
4	100 sea water + 1 KCl	39± 7.8	20	288	17
	sea water control	57±11	19.2	216	16

* The numbers indicate cc. of 5/8 M solutions.

the survival times of specimens in sea water or salt mixtures similar to sea water and it is now evident that many tests have to be run with each solution, including a parallel series of sea water controls with animals collected at the same time and place, and the probable error and coefficient of variation worked out (Table I). The probable error is calculated from Peter's formula and the coefficient of variation is expressed as 100 × P. E. divided by the mean. The earlier results with toxic solutions of single salts are clear-cut and show little variation, but the great variation in ternary salt solutions can be seen in the following sample giving survival times of 14 animals each in 100 cc. of sea water: 5.5, 5.5, 5.5, 5.5, 14.5, 14.5, 14.5, 21, 29.5, 31, 31, 53.2, 192 and 240 hours respectively.

Survival in Air and in Water at High Temperatures

Ligia baudiniana dies in about one hour if placed in aerated sea water at 38° C. (Table II), but at this temperature in air (over filter

TABLE II

Survival times in sea water and in air at high temperatures

Sea water			Air		
Temperature	Average survival	No. specimens tested	Temperature	Average survival	No. specimens tested
°C.	*minutes*		°C.	*minutes*	
38	61.8 ± 4.7	15	42	52.7 ± 5.7	14
44	7.6 ± 1	8	44	20 ± 2	19
50	0	8	45	11.5 ± 3	6
			50	3 ± 0.3	16

paper moistened with sea water) it survives so long that the longevity could not be determined. At 42° C., however, specimens in air die in slightly less than an hour and 50° C. is rapidly fatal in both water and air. If a specimen is slowly heated in 50 cc. of sea water with a temperature rise of about ½° C. per minute, the gill-beat becomes irregular at 39 – 40° C. and death occurs at 40–42° C. The Arrhenius equation, velocity $\alpha e^{-\mu/RT}$, was applied to the data for the rate of killing in air and yields a value of μ, the temperature characteristic, of 56,400 calories.

Temperature Characteristics for the Gill-Beat in "Heavy" Sea Water

The flapping of the pleopods is regular over a temperature range of about 10–38° C., but the frequency is too great above 28° for accurate timing with a stop-watch. In a typical specimen the time for ten beats increases from 2.5 seconds at 26° to 7 seconds at 10°. The gill-beat begins to be irregular at 10° and stops below this critical temperature. After exposure to 10° or below the frequency remains lower than normal when the temperature is raised (Fig. 1). Therefore, the data used in calculating the temperature characteristic were obtained from readings taken as the animal was cooled from 27° to 10°. As will be seen from Fig. 1, the readings on the upward temperature curve are all lower than those in the downward direction. For the heavy water experiments 2 cc. of sea water were evaporated down and the salts redissolved in 2 cc. of 96 per cent D_2O (specific gravity 1.1037). The same procedure was used for the controls except that the salts

from the evaporated sea water were taken up in ordinary distilled water. The frequency of the gill-beat immediately falls in "heavy" sea water. Thus at 20° C. a typical determination for the time for ten beats was 3.8 seconds in H_2O and 4.5 seconds in D_2O. In Fig. 1 it will be seen that this lower frequency obtains throughout the entire temperature range in heavy water and that exposure to 10° is followed by a further reduction in rate as in ordinary sea water.

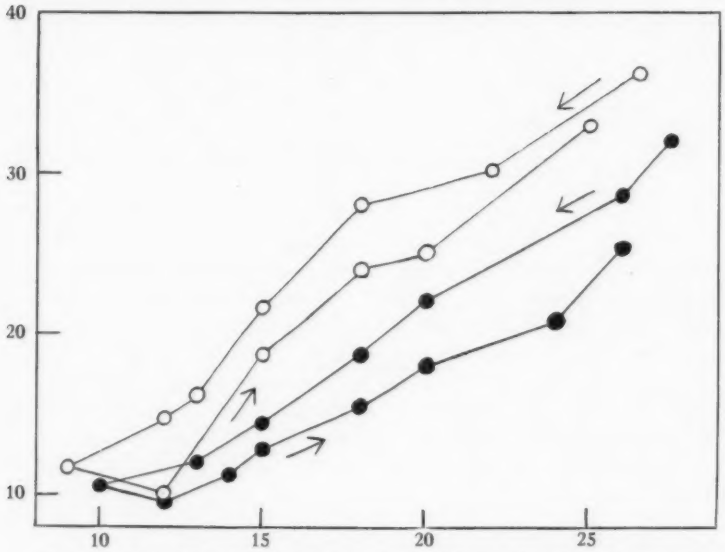

FIG. 1. Rate of beating of the pleopods of *Ligia* as a function of temperature. Ordinates, rate expressed as 100 divided by the time for 10 beats. Abscissæ, temperature in degrees centigrade. Open circles represent a specimen in ordinary sea water. Black dots represent a specimen in sea water containing 96 per cent heavy water. The rate was taken at the higher temperature first and then at progressively lower temperatures to about 10° where the beat becomes irregular. Then the temperature was raised, as indicated by the arrows. Each point is the average of five determinations of the time for 10 beats. Each run from 28° to 10° or 10° to 28° required about 40 minutes.

The data for both ordinary and "heavy" sea water are plotted in Fig. 2 according to the Arrhenius equation

$$ln\frac{R^2}{R^1} = \frac{\mu}{2}\left(\frac{1}{T^1} - \frac{1}{T^2}\right),$$

where R^1 is the rate at T^1 and R^2 is the rate at T^2. It appears that the slope of the line changes sharply at 16.5° C. in H_2O sea water and at 19.5° C. in D_2O sea water. The frequency is greatly reduced in heavy

water but it is interesting to observe that the lines for both media are parallel. In ordinary sea water the temperature characteristics are: 16,000 calories for the interval 10–16.5° C. and 8,400 calories between 16.5 and 27° C. For "heavy" sea water the μ values are; 15,000 calories, 10–19.5° C. and 8,400 calories between 19.5° and 27° C. The significance of these findings will be discussed in the sequel. There were no signs of injury during the hour and a half run in heavy water and the reduction in frequency of the gill-beat was reversible after return to ordinary sea water. Thus a specimen in which the time for ten beats was 5.5 seconds at 17° in D_2O showed a typical rate of ten beats in 4.2 seconds at 17° after return to sea water.

It will be noted in Fig. 2 that the scatter of the plotted points is

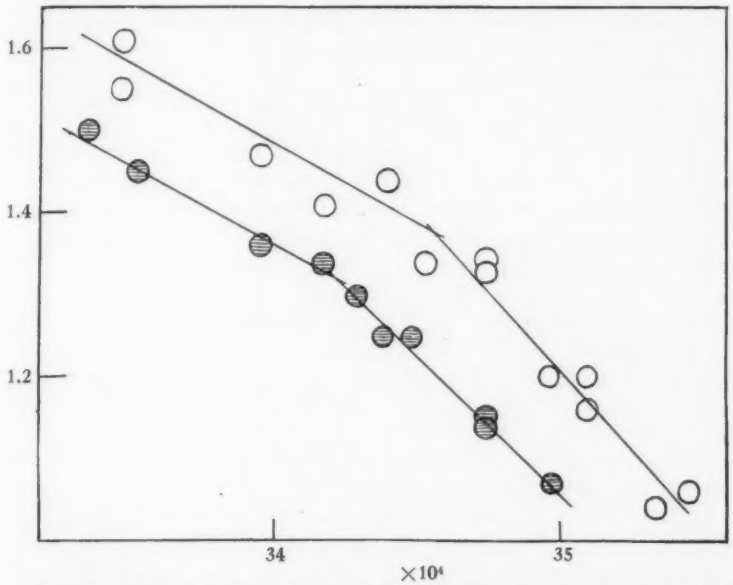

FIG. 2. Relation between the log of the rate of gill-beat in *Ligia* (ordinates) and the reciprocal of the absolute temperature (abscissæ). Open circles represent data from two specimens in ordinary sea water. Shaded circles represent readings from two individuals in sea water containing 96 per cent heavy water. In the upper graph the lines intersect at 16.5° C., in the lower at 19.5° C. (See text.)

much greater in H_2O sea water than in D_2O sea water. Owing to the scarcity of concentrated heavy water, the determinations were made from two specimens. Although many animals were used in ordinary sea water, the data from only two typical specimens are plotted in Fig. 2 as a basis for comparison with the D_2O results. This procedure is

justified by the fact that in ordinary sea water the temperature characteristics are the same as those obtained by Numanoi (1935), who used hundreds of specimens of the closely allied *Ligia exotica*. The specimens used in the 96 per cent D_2O measured 13 and 14 mm. in length (not counting uropods and spines) which were large in comparison to the 2 cc. of water, so that the concentration in the tissues was probably about 80 per cent heavy water.

Discussion

Evidence is accumulating that the inability of *Ligia* to survive when immersed in sea water is not entirely determined by the relative proportions of the chief cations. However, it is interesting to note that a reduction by half of the Mg content of artificial sea water permits greater longevity in that medium but a synthetic sea water of greater sustaining power than natural sea water has not yet been found.

The studies of the rate of killing of the isopod at high temperatures indicate an abrupt transition from the rate of killing curve to the ordinary curve of life duration as found by Brown and Crozier (1927) in cladocerans. The lethal temperatures for *Ligia* in Bermuda do not appear to be higher than those for organisms in the temperate zone, but the minimum temperature for the gill-beat, 10° C., is four degrees higher than that of *Ligia exotica* in Japan (Numanoi, 1933). The high temperature characteristic for the rate of killing of *Ligia* at high temperatures is similar to values obtained from such processes as the coagulation of proteins by heat (Buchanan and Fulmer, 1930, p. 52). The more rapid lethal effect of high temperature in water than in air is a common phenomenon and probably not connected with the greater longevity of *Ligia* in air at ordinary temperatures (25–28°).

The temperature characteristic of 16,000 calories obtained for the gill-beat is one repeatedly encountered for respiratory processes (Numanoi, 1933) and is associated with the catalysis of oxidation by iron (Crozier, 1924). The μ values obtained by Numanoi (1933) for the gill-beat of *Ligia exotica* in Japan, i.e., 15,200–17,000 calories between 6–16° C. and 7,000–8,200 calories between 16 and 30° C., are precisely the same for *Ligia baudiniana* in Bermuda. These two values occur together frequently for respiratory processes.

The application of the Arrhenius equation to the data for the gill-beat in heavy water should throw light both on the mechanism of action of the heavy hydrogen isotope and on the validity of the Arrhenius equation in biological systems. Crozier (1935) has recently discussed modifications of temperature characteristics from which it is clear that if the several discrete magnitudes of μ persist in spite of

modifications of the temperature graph, it may be concluded that the temperature characteristic represents the molecular energy of activity of a specific chemical process. Now it will be seen in Fig. 1 that in spite of the great reduction in frequency by heavy water, the rectilinear relation is not destroyed and the same discrete values of μ can be recognized as for the process in ordinary water. Not only so, but the data from the heavy water series fit the equation even more closely in that the points all lie on the line.

It appears that the heavy water reduces the frequency of the gill-beat without changing the fundamental regulating mechanism. A similar effect is seen in the action of adrenalin on the heart (Crozier, 1926), in which the frequency is greatly increased but the temperature characteristic is not changed. Urey and Teal (1935), when discussing the energies of activation of chemical reactions in heavy water, point out that no difference in the activation energies need be expected in reactions in which hydrogen is not directly involved. On the other hand, the reaction between deuterium and bromine to form DBr has an activation energy of 19,870 calories which is 2,100 calories higher than that for the reaction $H + Br = HBr$, the difference being about equal to the difference in the zero point energy of the two hydrogen isotopes.

It remains to be seen if there are biological processes in which the temperature characteristic is changed greatly in heavy water. The rate of pulsation of the contractile vacuole in *Paramecium* (Barnes and Gaw, 1935) has three values of μ over the temperature range in ordinary water i.e., below 16°, 24,000; 16–22°, 17,000; and above 22, 14,000; but in heavy water the value is 22,000 over the entire range. The rate of pulsation of the turtle's auricles (unpublished data), like the gill-beat in *Ligia*, yields a μ value of 16,000 in both D_2O and H_2O. It is hoped that further temperature studies in heavy water will afford new and significant information concerning the regulation of physiological processes.

The effect of heavy water on the rate of the gill-beat is equivalent to a drop in temperature of 3–4° C. This is also seen in the critical temperature or point in the graph (Fig. 2) where the slope of the line changes. The line breaks at a point 3° higher in the plot for the heavy water experiment. It is proposed that the effect of heavy water on a physiological process should be expressed as an "*equivalent drop in temperature*" of a specific number of degrees. This is similar to a new method of expressing the configuration of the water molecules in a dilute solution of strong electrolytes proposed by Bernal and Fowler (1933)—the "structural temperature" or that temperature at which

pure water would have effectively the same inner structure as the given solution.

Summary

1. The survival of *Ligia* in artificial sea water is doubled if the Mg content is reduced by one half.

2. In spite of the fact that *Ligia* cannot survive long periods in natural sea water, no artificial sea water has been found to be a more favorable medium.

3. Exposure of about one hour to sea water at 38° C. or to moist air at 42° C. is fatal to *Ligia*.

4. In natural sea water the beating of the pleopods of *Ligia baudiniana* yields temperature characteristics of 16,000 calories between 10 and 16° C. and 8,400 calories between 16 and 28° C. These are typical of respiratory processes.

5. The reduction in the frequency of the gill-beat in sea water containing 96 per cent heavy water is equivalent to that produced by a drop in temperature of about 3–4° C. Similarly the critical temperature in heavy water is shifted three degrees higher.

6. In sea water containing 96 per cent heavy water the gill-beat yields temperature characteristics of 15,000 calories between 10 and 19 and 8,400 calories between 19 and 28° C.

7. It is concluded that the lower energy content of heavy water slows down the beating of the gills but does not change the fundamental regulatory mechanism.

It is a pleasure to express my gratitude to Dr. J. F. G. Wheeler, who placed the facilities of the Bermuda Station at my disposal.

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THE BIOLOGY OF CALANUS FINMARCHICUS IN THE GULF OF MAINE AND BAY OF FUNDY

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Introduction and Methods

The problem considered in the present paper concerns the production and dispersal of *Calanus finmarchicus* in a partially enclosed body of water. There have been two principal objectives; first, a determination of the breeding seasons, annual number of broods, rate of growth and length of life; second, an evaluation of different spawning areas, and dispersal of eggs and larvæ.

Monthly cruises were made from July 28, 1931 to September 29, 1932 in the Bay of Fundy and from April to September, 1932, in the inner part of the Gulf of Maine, with one cruise in the outer Gulf and along the south coast of Nova Scotia, yielding in all a total of 838 zoöplankton samples (Fig. 1). In August 1931 one line of stations was taken in the Gulf.[2]

For convenience in description, the region has been subdivided into four areas shown in Fig. 1. For the sampling of eggs and immature stages, the pump was used during the summer, a volume of 250 liters of water from each of four levels, 0, 10, 30, and 50 meters being filtered at each station. Analyses were made by the Sedgewick-Rafter counting method, and the number of individuals per cubic meter calculated. In determining horizontal distribution, in the present report, the value taken for each station represents the mean of the four levels. Until weather conditions permitted the use of the pump (end of June), half-meter nets (two meters in length) of No. 10 silk were utilized. The hauls were of 15 minutes duration and were oblique from 50 meters to the surface, the distance being divided into six steps at intervals of 10 meters each. The counts were made by the same method used in the pump samples, but the number of organisms per minute of towing was taken as the unit of measure.

Importance

Calanus finmarchicus is the most abundant animal in the Gulf of Maine, occurring offshore throughout the year and well into inshore

[1] Contribution No. 85.

[2] Investigations for the International Passamaquoddy Fisheries Commission.

waters except during the warm summer months when its distribution is somewhat variable. In Frenchmans Bay, with temperatures as low as 7–9° C. persisting in the lower levels, it was found abundant throughout the summers of 1929, 1930, and 1932, but in shallower or more turbulent estuarine areas such as Passamaquoddy Bay, where the

FIGURE I

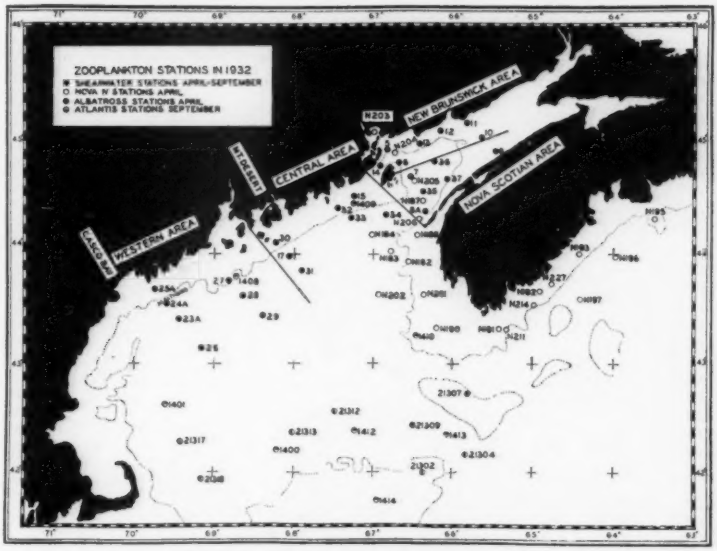

FIG. 1. Zoöplankton stations in 1932.

whole water mass responds more rapidly to seasonal warming (10–12° C. after August 1), *Calanus* was comparatively sparse between June and October.

Its importance in the region is indicated in Tables I and II giving the relative percentage by number of *C. finmarchicus* to the total zoöplankton population. Within the Bay of Fundy, *Calanus* was found most abundant at all times in the vicinity of the deep basin, with year values increasing upon approaching the entrance. Thus, as indicated below, at Station 5 in the Quoddy region the mean for the year was 32.4 per cent, at Station 6 east of Grand Manan 37 per cent, and at Station 8A near the mouth on the Nova Scotian side, 41.8 per cent.

In the Gulf of Maine the highest values occurred in the central area where production of many of the pelagic species breeding during the summer appears to be largely unsuccessful. Here *C. finmarchicus* ranged from 40 per cent to 90 per cent, except in April when locally spawned larvæ of *Balanus balanoides* dominated the hauls.

Bigelow (1926, p. 199) found that in the Gulf of Maine *Calanus* averaged approximately 55–60 per cent of the total copepod content. In 1932 it averaged 60.4 per cent of the zoöplankton population in May with a total copepod content of 77.2 per cent and 39.9 per cent for the period April to September. In the eastern Atlantic Ruud's (1929, p. 11) records indicate that off Möre in 1926 and 1927 *C. finmarchicus* formed about 13–15 per cent of the copepod population in March and rose to 63.4 per cent following vernal augmentation. The average for the period March–July during the two years was approximately 39 per cent.

TABLE I

Relative percentage by number of *C. finmarchicus* to the total zoöplankton population, 1931–32, Bay of Fundy.

1931–32	Bay of Fundy			
Month	Mean	Station 5	Station 6	Station 8A
September	28.5	17.5	6.7	61.4
October	23.4	23.4	—	—
November	31.3	36.1	34.6	23.3
December	37.9	61.2	34.2	18.2
January	25.9	25.9	—	—
March	61.6	73.2	57.3	54.4
April	34.8	49.7	36.6	18.1
May	41.0	4.8	56.7	61.5
June	36.4	15.5	42.5	51.2
July	29.0	29.0	—	—
August	31.4	20.0	27.8	46.3
Mean for year	35.5	32.4	37.0	41.8

It would thus appear that in the Gulf of Maine *C. finmarchicus* forms a more important member of the zoöplankton population than in the region off Möre, although at no time were eggs found in such quantities as reported from certain areas between Norway and Iceland, where Damas (1905, p. 12) found them at times comprising practically the entire haul. In the Gulf of Maine at the peak of June spawning (1932) eggs of *Calanus* were the dominant form, but were everywhere outnumbered by the combined eggs and larvæ of other species. In

the Bay of Fundy, also, Willey has commented (1921, p. 189) on the absence of *Calanus* eggs in any such relative abundance as reported by Damas. However, the importance of this species is evidenced by the manner in which the total volume of zoöplankton follows the fluctuations in the *Calanus* content (Fig. 2), a condition also noted by Ruud (1929, p. 12) for the Möre area.

Regional Distribution of the Adult Stock

The quantitative distribution of adult *C. finmarchicus* (including late copepodite stages) in the Gulf of Maine as described by Bigelow

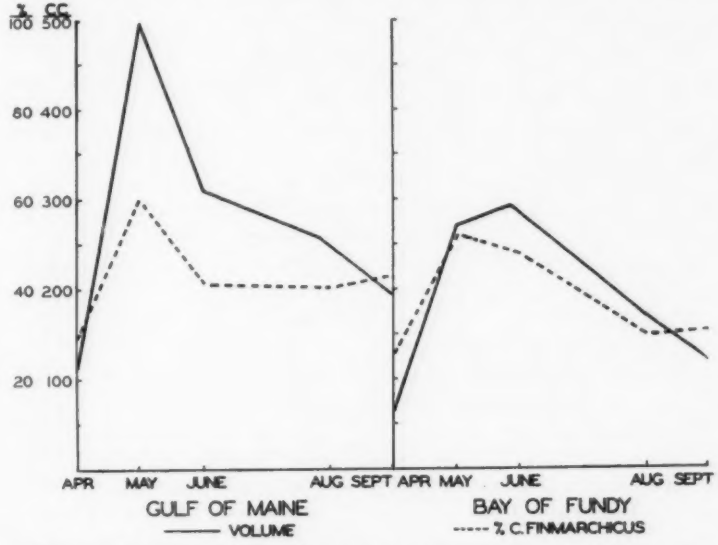

Fig. 2. Volume of zoöplankton and relative percentage by number of *C. finmarchicus* (adults and late copepodites) in the Gulf of Maine and Bay of Fundy in 1932.

(1926, p. 188) accords so closely with present data that little need be added here. Although almost everywhere abundant, Bigelow found marked horizontal variations. Together with other boreal plankton animals the smallest numbers were found in 1931 and 1932 in the Bay of Fundy, particularly on the New Brunswick side, and along the adjacent coast of Maine to Penobscot Bay. However, although the values were uniformly low, due to equally small numbers of other boreal zoöplankton species, *C. finmarchicus* is relatively almost as

important a member of the population in this region as elsewhere in the Gulf. The largest numbers in the Bay of Fundy occurred in the deep basin and along the Nova Scotian coast; in the Gulf of Maine, at the outer stations.

Production and Dispersal

Propagation of *Calanus finmarchicus* has been found to vary greatly in different localities, both in the rate of development and the number of broods. Available records indicate that in the southern portion of its range development is relatively rapid and spawning may begin as early as January. Proceeding northward into colder water, the period of vernal augmentation becomes increasingly later, development slower, and there are fewer broods.

In the Clyde Sea, a fairly enclosed basin with a mean annual temperature of over 10° C. and minimum holothermic temperatures in the spring of 5–7° C. (Chumley, 1918, p. 14),[3] eggs and nauplii of *C. finmarchicus* were found by Nicholls (1933, p. 89) in January,

Table II

Relative percentages by number of *C. finmarchicus* to population of total region of Bay of Fundy, and of Gulf of Maine in 1932.

1932	Total region	Bay of Fundy	Gulf of Maine		
			Total gulf	Western area	Central area
April	25.7	24.6	26.8	34.9	18.7
May	56.4	52.4	60.4	66.0	54.8
June	44.7	47.5	41.9	44.4	39.4
August	35.6	29.8	41.4	39.4	43.3
September	37.3	30.7	43.9	43.2	90.2
April–September	39.9	37.0	42.9	45.6	49.3

followed by three well defined breeding periods, February–March, April–May, and June–July, with additional subsidiary broods of lesser importance in July and August. These broods represent successive generations, the adults dying off after spawning.

In Norwegian waters off Möre, Ruud (1929) found propagation beginning in late March to early April, the maximum having passed by May both in 1926 and 1927. A second smaller maximum appeared in June and continued until late July. His records also indicate that spawning takes place earlier farther south and proceeds northward along the Norwegian coast where augmentation in spring and early

[3] In the Gulf of Maine there is a mean annual temperature of 7–8° C. and a minimum holothermic temperature of about 2° around the margin.

summer (April–June) had previously been reported (Gran, 1902; Damas, 1905; Damas and Koefoed, 1907, etc.).

In the region of Iceland, Paulsen (1906, pp. 19–20) reported the principal propagation season of *C. finmarchicus* to be from March until June with a possible second reproduction period later. At the meeting point of the Atlantic and North Polar currents, south of 67° N, the season is in late June (Damas, 1905), and east of Greenland, in late July (With, 1915).

In the Gulf of Maine and Bay of Fundy previous investigations have shown the stock of *Calanus* to be at a minimum in late February and early March, at which time regional differences would be expected to be least. Vernal augmentation begins in late March and April (depending on the locality) and varies somewhat from year to year. Early nauplii were abundant in Massachusetts Bay by April 7 in 1925 (Fish, 1928, p. 286). In coarse net hauls where the effect of production would not be noted until the new stock had reached copepodite stages, Bigelow (1926, p. 192) observed in 1920 a slight increase from March to April in the coastal zone between Cape Cod and Mt. Desert, a considerable increase in Massachusetts Bay (× 400), and in the western basin about the same average number as in February and March. His data of several years led him to conclude that probably the "multiplication of *Calanus* does not proceed so rapidly in the northern parts of the Gulf, though it may commence there as early as mid-April" (Ibid, p. 194). There was also evidence that breeding probably continues actively through June, and an increase in the numbers of *Calanus* in September suggested a second but less productive season in early autumn (Ibid, p. 207).

To the north Willey (1919) found evidence of vernal propagation over a broad area between Nova Scotia and Newfoundland. At the eastern end of St. Pierre Bank in 1915 *Calanus finmarchicus* in copepodite stages II–V was taken on June 24 and again on July 27. Stages III–VI were also obtained south of Cabot Strait on July 27 and in the Laurentian Channel on July 28.

Distribution of Eggs and Larvæ in 1932

An analysis of propagation in the Gulf of Maine and Bay of Fundy is complicated by the progressive delay in seasonal response from west to east (Fig. 3) combined with an active cyclonic drift, which renders it difficult to ascertain the source of larvæ taken in any particular locality. Fortunately an average difference of approximately one month in the time of spawning in the eastern and western parts of the Gulf made it possible to distinguish two breeding stocks, and, through the distribution of dominant developmental stages in successive

months, trace in a general manner the rate of growth and subsequent dispersal in each.

April.—Spawning must have begun in March, and by late April had terminated over the greater part of the Gulf. The more advanced

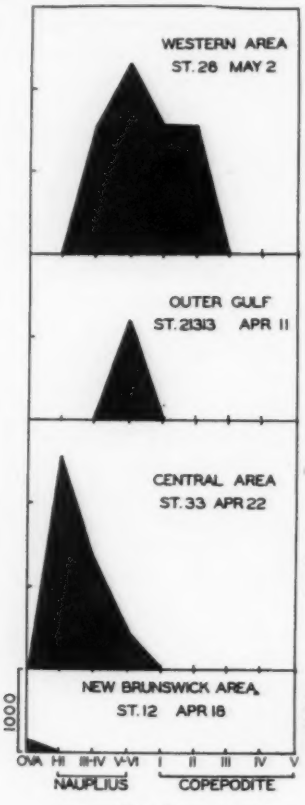

FIG. 3

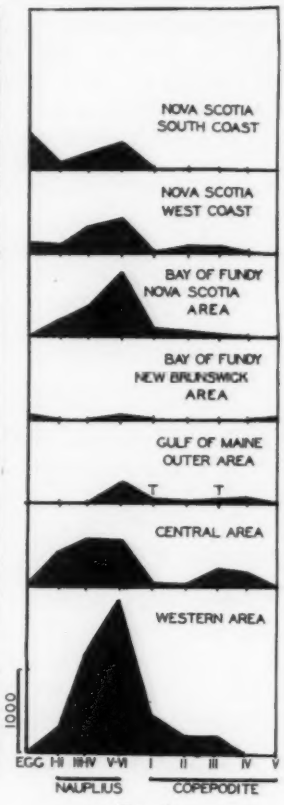

FIG. 4

FIG. 3. *Calanus finmarchicus*, showing progressive delay in seasonal response to the eastward, and evidence that the larval stock in the outer Gulf may have originated in the western area.

FIG. 4. *Calanus finmarchicus*, showing nauplius stages IV–VI dominating throughout the region in April 1932. (Average of all stations in each area.)

larvæ had then reached the third and fourth copepodite stages, and were rather widely distributed in relatively small numbers. Nauplius stages IV–VI predominated in the Casco Bay region where production

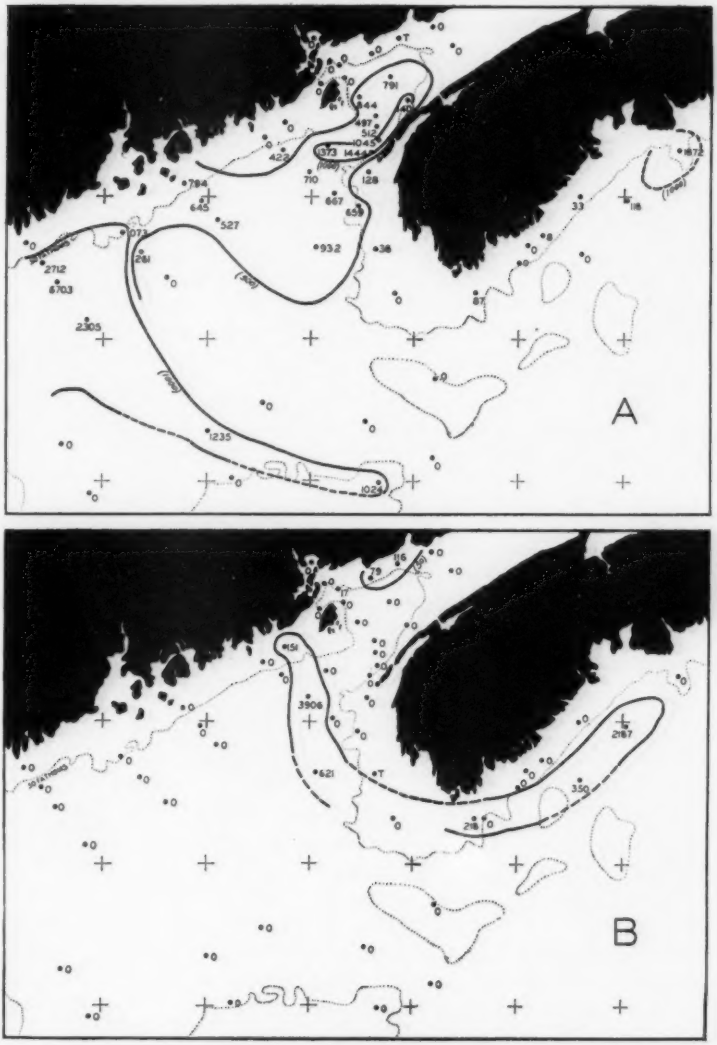

FIG. 5. Distribution of (*A*) nauplius stages V–VI of the western stock, and (*B*) eggs of the eastern stock in April 1932. Number per minute of towing.

was so much more extensive than elsewhere that its contributions outnumbered locally produced larvæ over the greater part of the Gulf and Bay, a condition which continued throughout the summer. As it was possible to make but one series of observations in the outer Gulf, the duration and extent of propagation in that area could not be determined, but the distribution of larvæ, consisting of nauplius stages V–VI with a small number of copepodite stage III and no intermediates, would suggest the western coastal area as the principal source. However, a local concentration of nauplius stages V–VI off Nova Scotia offers positive evidence that in outside waters the season was as far advanced off Halifax as in the region of Casco Bay. Between these two areas, along the south and west coasts of Nova Scotia, the season appeared to be less advanced, more closely corresponding to the eastern part of the Gulf where spawning had not yet ceased.

TABLE III

Maximal numbers of *Calanus finmarchicus* in various developmental stages in the western area of the Gulf and the Bay of Fundy, May, 1932.

Locality	1932	Ova	Nauplius			Copepodite				
			I–II	III–IV	V–VI	I	II	III	IV	V
Western area (Station 26)	May 31			1029	515	1029	3603	8234	4117	2058
Bay of Fundy (Station 36)	May 22	669	1338	6021	8697		1338	669		

Eggs of the eastern breeding stock were found in varying abundance in the central area, the Bay of Fundy, and off the west and south coasts of Nova Scotia, but not west of Mt. Desert or in the vicinity of Georges Banks.

May.—By the latter part of May, as indicated in Table III, the maximum numbers of larvæ from the western area were in copepodite stage III, and the local crop east of Mt. Desert, in nauplius stages V–VI, the latter corresponding to conditions in the western area a month earlier. In Fig. 6A late larval stages are seen to extend eastward from the rich Casco Bay region in a band whose inner margin parallels the 100-meter contour. They also penetrated in a tongue-like wedge into the Bay of Fundy along the Nova Scotian side, and show as a distinct second maximum in curves from both the Fundy and central areas (See Fig. 8, May sections off Cape Spencer, and Mt. Desert; also Table III, Station 36). These late copepodites of *Calanus finmarchicus* now dominated the total population and accounted largely for the tremendous rise in the volume of zoöplankton from April

to May (Fig. 2).[4] Due to dispersal from the western area volumes everywhere increased, the increase being least noticeable in areas where

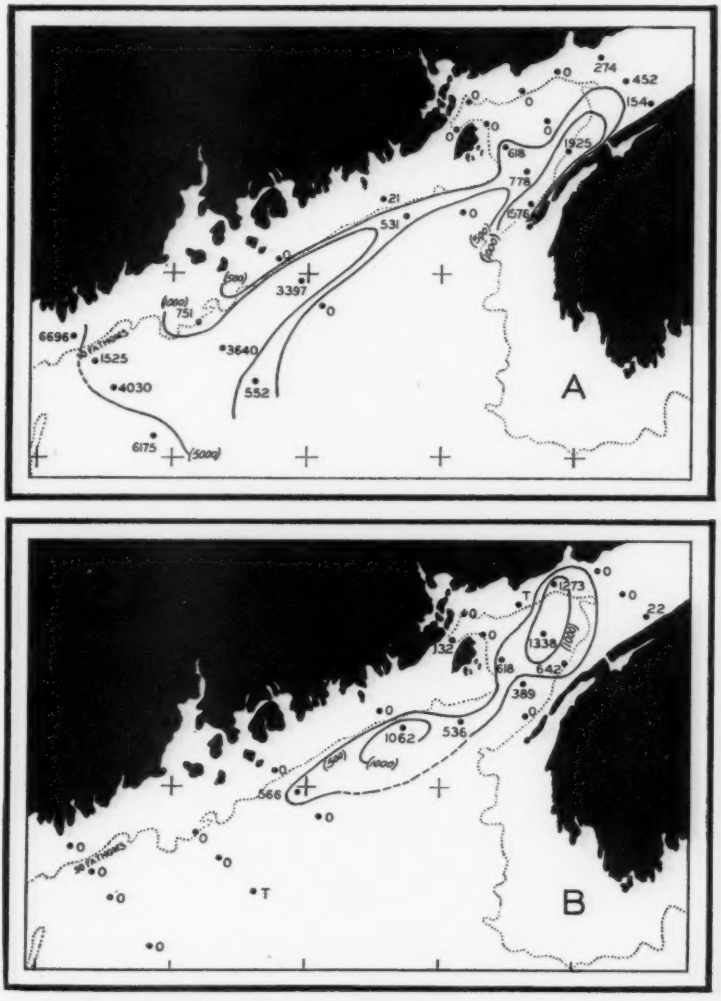

FIG. 6. Distribution of (*A*) copepodite stages IV–V of the western stock, and (*B*) nauplius stages I–II of the eastern stock in May 1932. Number per minute of towing.

[4] Bogorov (1933) records an increase in the biomass (weight) of almost 60 times from copepodite stage I to stage VI.

the immigrant stock was not supplemented by a significant amount of local propagation. Particularly in the eastern coastal and New Brunswick regions, individuals of the local brood were not of sufficient size to be taken in the coarse meter nets in May.

Although late nauplii dominated in the eastern Gulf stock, some spawning must have continued well into May in the coastal waters east of Petit Manan, because early nauplii (I–II) were still numerous in this region between May 18 and 24 (Fig. 6*B*).

June.—The first western crop had matured and were spawning by June 20. As eggs were everywhere abundant (Figs. 7*A* and 8), although the eastern brood was still in copepodite stages (Fig. 9), the parent stock in all probability consisted almost entirely of individuals dispersed from the western area. In the eastern basin it is possible that the adults breeding at this time may have been reinforced to some extent from production centers outside of the Gulf, a possibility suggested by Bigelow's (1926, p. 209) records of a swarm of adults in this region on May 6, 1915, and the fact that in 1932 the largest single capture of eggs (25,321 per minute) was made in the eastern basin in June.

The eastern spring brood, in copepodite stages in June (Fig. 7*B*), were most numerous off Penobscot Bay and along the course of the drift into the Bay of Fundy. Elsewhere in the Bay and over the greater part of the central area, no copepodite stages were found, indicating that this brood suffers heavy mortality and only those produced in or transported as far as the western part of the central area survive.

August.—On August 11 in the Casco Bay region, after an interval of 42 days, the product of June spawning of the western stock, now in copepodite stages (III predominating), had been largely dissipated and the relatively few taken were distributed along the outermost stations from Casco Bay to the eastern basin. Elsewhere in the Gulf and Bay their numbers were extremely sparse.

Contrasted with copepodites of this second western crop, eggs and early larvæ, assumed to represent spawning by the first eastern brood, were concentrated nearer the coast. Early nauplii were most abundant in the Bay of Fundy (Station 7) and central area, with smaller numbers occurring westward at the inner stations to Casco Bay.

September.—It was not possible to obtain data from the Gulf of Maine in September, but stations in the Bay of Fundy yielded small numbers of eggs, early nauplii (stage I) and copepodites in stage II. The latter were probably the product of August spawning by a second eastern brood and the former, in view of the absence of eggs and early

larvæ along the New Brunswick coast and their concentration in the course of the drift along the Nova Scotian coast and over the deep basin in the Bay of Fundy on September 15, suggest a third generation of the western stock entering from the Gulf.

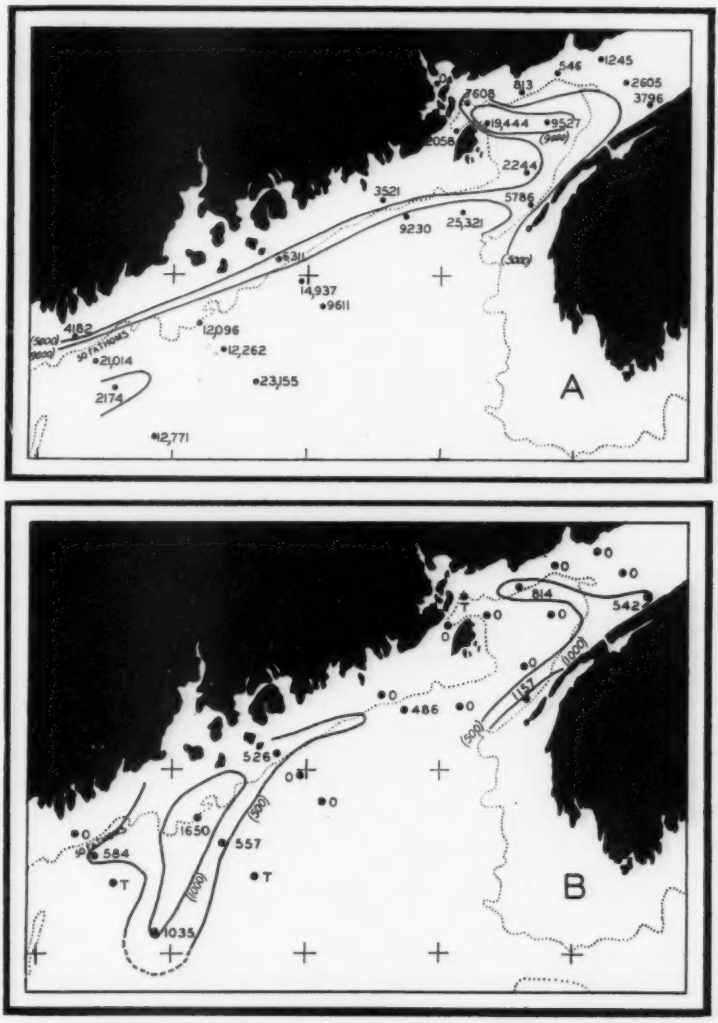

FIG. 7. Distribution of (*A*) eggs of the western stock, and (*B*) copepodite stages I–V of the eastern stock in June 1932. Number per minute of towing.

Comparison of Results in 1931 and 1932

In 1931 but one set of observations was made in the Gulf and Bay (August 21–September 5), corresponding stations being taken about two weeks later than in 1932 (August 8–21).

The season appears to have been about the same in the western area during the two years because a maximum of eggs between August 11 and 14 in 1932 would undoubtedly have hatched by August 21 when early nauplii were found predominating in 1931.

East of Mt. Desert, as the relative percentage of eggs and nauplii were much the same in the Nova Scotian and New Brunswick areas on August 17–21 in 1932 as on September 1–5, the spawning season would appear to have been about two weeks later in the previous year. Not having hydrographic data from the eastern basin of the Gulf for 1931, it is not possible to suggest a reason for the retarded season that year. Temperatures within the Bay of Fundy in 1931 and 1932 were not substantially different at this time.

Discussion and Conclusions

Throughout the Gulf of Maine and Bay of Fundy propagation begins in the spring and is followed by a succession of generations until autumn. The marked regional differences in the time when vernal augmentation begins are reflected through succeeding generations of that summer, irrespective of where the stock may become distributed. Thus by the time (June) that the spring crop from the western area has matured and spawned, it has become so generally distributed that eggs appear almost simultaneously throughout the Gulf and Bay (Fig. 8). Again, in July and August, when the smaller eastern vernal crop spawns, eggs appear in even greater abundance in the western area than in the region where the stock originates.

Propagation of the Stock Originating West of Mt. Desert in the Spring

The seasonal distribution of eggs and larvæ indicate two clearly defined spawning periods, March–April and June–July with a possible third in September. It is probable that most of the June–July brood remains in copepodite stages to form the bulk of the winter stock (maturing the following spring), but some appear to pass through the final moult and spawn in September.

The maximum numbers of larvæ of the first generation, which by late April in 1932 were in nauplius stages IV–VI, and in copepodite stage III thirty days later, had matured and were spawning by June 20.

The second generation is not as clearly traceable, but what are believed to have been members of the June brood were mainly in copepodite stage III forty-five days later on August 11 (Fig. 8, section *A*), and some became spawning adults by September 15 (p. 129).

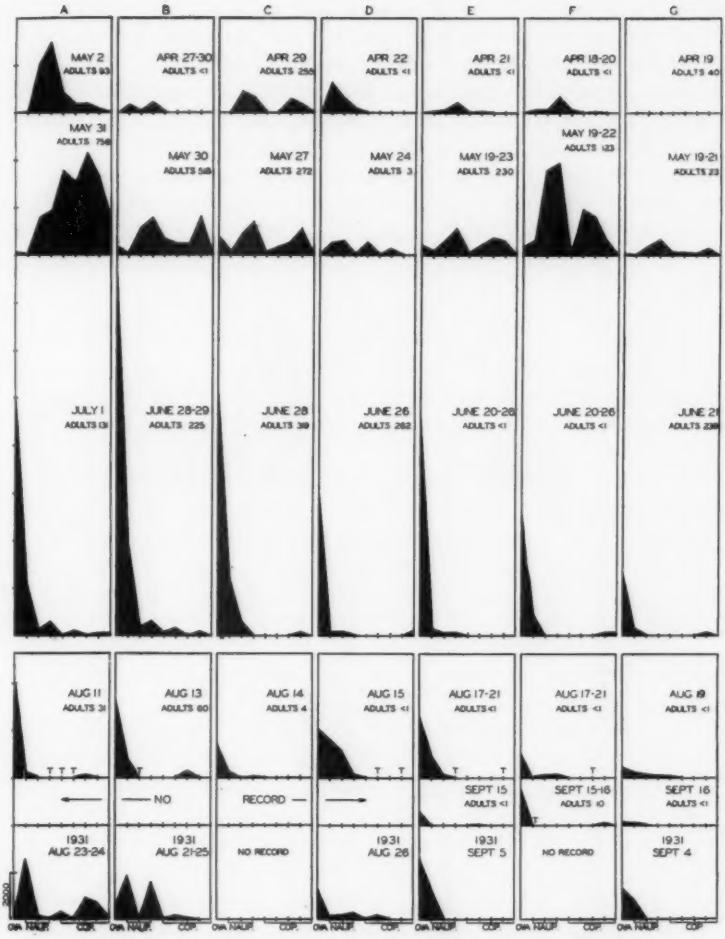

FIG. 8. Seasonal changes in the composition of the population of *Calanus finmarchicus* (developmental stages) in seven sections between Casco Bay and Cape Spencer.

Stages recorded as ova; nauplii I–II, III–IV, V–VI; copepodites I, II, III, IV, V.
Sections: *A*. Casco Bay (Stations 23*A*–25*A*, 26)
B. Penobscot Bay (Stations 27–29)
C. Mt. Desert (Stations 17, 30–31)
D. Moose Peak (Stations 32–33)
E. Passamaquoddy Bay (Stations 5–8*A*, 35)
F. Pt. Lipreau (Stations 13, 36–37)
G. Cape Spencer (Stations 9–11).
April–June: number per minute of towing.
August–September: number per cubic meter.

Propagation of the Stock Originating East of Mt. Desert

This region (Fig. 1) shows considerable local variation. In its western portion approaching Mt. Desert and on the Nova Scotian side of the Bay of Fundy (influenced by inflow from the outer Gulf), the spawning seasons of *Calanus* more closely approximated those to the west of Mt. Desert, while along the coast eastward from Petit

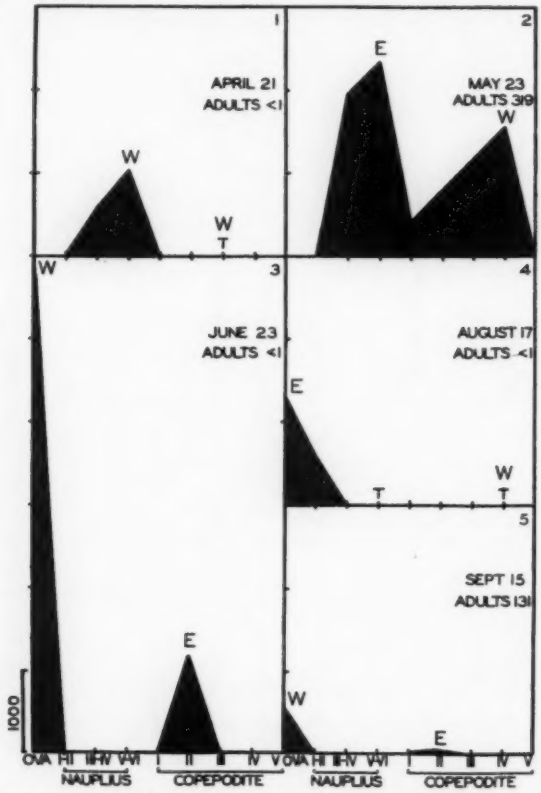

FIG. 9. Development of the western (*W*) and eastern (*E*) stocks as indicated at station 8*A* in the drift entering the Bay of Fundy in 1932.

Manan to St. John the seasons were progressively later. However, although evident, these local seasonal differences are relatively small, and for present biological purposes the waters east of Mt. Desert can be considered to comprise one region in which the maxima of corresponding stages of *Calanus* occur, as previously noted (p. 123), about four weeks later than in the western part of the Gulf.

There appear to be two less well defined propagation periods of the eastern stock, April–May and July–August. The first is indicated by eggs appearing along the New Brunswick coast on April 18 (Fig. 3) and the maximum of nauplius stages I–II in the vicinity of Petit Manan (Station 33) on April 22 (Fig. 3). Reaching nauplius V–VI a month later (May 18–24) and copepodite stages II–IV on June 20–26 (Figs. 8 and 9), it is presumed that August (8–21) eggs represent spawning by adults of this stock.

It was not possible to trace with certainty the outcome of July–August propagation because of the small number of individuals involved, but it would appear that the copepodites (stage II) entering the Bay of Fundy on September 15 (Fig. 9) were representatives of this crop. This second eastern brood of copepodites probably supplements the second and third (?) western broods to form the winter stock.

Rate of Early Development

In the Clyde Sea (Nicholls, 1933, p. 92) field and laboratory records have indicated a maximum of four weeks for the complete development of *Calanus finmarchicus*, a minimum of eleven days between the egg and first copepodite stage and intervals of three days for successive moults to stage V. A minimum of four days was required for the final moult to stage VI (adult).

Lebour (1916, p. 9), in laboratory experiments at Plymouth, found that nauplii appearing between April 17 and 24 reached copepodite stage V on May 19, having taken less than two months for development from the egg to the latter stage.

Off Möre Ruud (1929, p. 38) estimates that *Calanus finmarchicus* requires almost three months for its life cycle, allowing three to four weeks for embryonal development as indicated by Lebour (1916, p. 9). In 1926 there was about seven weeks between the maximum of nauplii and the maximum of stage VI.

In the Gulf of Maine the developmental period appears to be slightly less than in Norwegian waters, but, as observations at each station were taken at minimum intervals of one month, the rate of growth can only be approximated. In the case of the western stock, with an interval of 29–33 days between the maximum of copepodite stage III in May and the presence of eggs in June, followed by a period of 42–46 days before the next maximum of copepodite stage III, the complete cycle would appear to require from 71–79 days. Again, if eggs observed on September 15 (p. 129) can be considered to represent spawning by individuals incubating west of Mt. Desert from June 28 to July 1, and in copepodite stage III on August 11–14, then there

must have been a maximum period of approximately 78–81 days between the second and third broods of eggs.

The two generations of the western stock would thus agree rather well and suggest a period of about two and one-half months from the egg to the spawning adult. Intervals between maxima of different stages also indicate about one month for the development from nauplius I to copepodite I, one-half month from copepodite I to copepodite V, and one month for the final moult, maturation, and incubation of the eggs. Although requiring confirmation by laboratory experimentation, there would seem to be about five days between moults in the nauplius stages and three days in the copepodite stages.[5]

No attempt has been made to calculate the developmental period of the eastern stock, hatching shortly before April 22 in the central area (Fig. 3, Station 33), because the greater part of this brood apparently matured and spawned in July, between cruises, and eggs taken in August no doubt represent late season spawning. This is indicated by the large percentage of nauplii up to stages III–IV in the central area on August 15.

August 15, 1932	Ova	Naup. I–II	Naup. III–IV	Naup. V–VI	Cop. I–V
Central area (Station 33).....	3,390	1,507	2,450	377	T

The rate of development would appear to be substantially the same as in the western stock, which is not surprising in view of the evidence that those members of the eastern spring brood reaching maturity probably entered the western area in early stages.

Rate of Late Development

As there was an interval of about one month between the maximum of late copepodite stages III–IV (May 27–31) and the next maximum of eggs (June 28–July 1) in the area west of Mt. Desert, it would appear that the time required for the ripening of the gonads and fertilization, after the moult to stage VI (adult), is probably about three weeks. A similar interval is suggested by the June brood if copepodite stages III–IV from the western region (August 11–13) formed the parent stock of early eggs entering the Bay of Fundy on September 15–16 (Fig. 8) in 1932. Nicholls (1933, p. 95) found that, having moulted to adults, there is a lag of from three weeks to one month before the next maximum of eggs in the Clyde Sea.

[5] At Station 8*A* copepodite stage II predominated on May 15, and copepodite stage IV on May 23, nine days later.

Data on the interval between the final moult and the time of spawning in the winter stock are available only for the Bay of Fundy. Here the period must be considerably longer than in succeeding generations in the Gulf. The winter population, almost entirely in late copepodite stages in November, is indicated in Table IV and also in a haul by Willey in Passamaquoddy Bay (1921, pp. 187–8) on November 2 in 1916 when copepodites in stage V formed 46 per cent and adult females only 2 per cent of the total macrocalanid plankton. By February 23 in 1917 adult males and females formed 47 per cent and copepodite stage V, 36 per cent. In 1932, as shown below, the winter stock in this area had almost completed moulting (97.6 per cent adults) by March 21 and a considerable proportion of the females contained developing eggs. Yet no eggs were found in these waters on April 15, and spawning had apparently just begun on May 5.

TABLE IV

Winter population of *C. finmarchicus* in Bay of Fundy. Percentages by number of various developmental stages to total zoöplankton population.

Date	Copepodite		Adult	
1931–1932	Stage IV	Stage V	♀	♂
November 16	3.8%	96.2%	0%	0%
March 21	0%	2.4%	92.9	4.7

Relative Abundance of Adults

Accurate figures on the relative number of adults and individuals in developmental stages are difficult to obtain because the former cannot be sampled in a representative manner with the apparatus required for eggs and early larvæ. Copepodite stages IV and V, however, appear to have been sampled efficiently with both the coarse and fine nets, and by taking the fine net counts and applying the meter net data on the relative percentage of these stages to adults, it has been possible to obtain roughly comparable figures for the latter. Similar figures have been obtained for August and September using pump and meter net data but the error may prove to be greater in this case if it is found that late larvæ, particularly copepodite stage V, cannot be sampled representatively with the pump. There is some evidence that this might be true, in which event the mean number of both adults and late copepodite stages in the total region would be somewhat larger than indicated in Table V.

Mortality among Maturing Adults

In the Gulf of Maine during the interval between the maximum of late copepodite stages in late May and the maximum of eggs in June, there occurred the highest rate of mortality observed in zoöplankton during the year, the decline in the relative percentage of *Calanus* exceeding even the tremendous decline in the total volume of animal plankton (Fig. 2) at a time when the spring stock of this species, just reaching maturity, might have been expected to substantially increase the total plankton volume (p. 128).

A similar decline in *Calanus* in the Clyde Sea (where, as in the Gulf of Maine, the maximum of eggs coincides with the minimum of adult females) has been interpreted by Nicholls (1933, p. 95) to indicate that the period of maturation forms a very critical time during which the rate of mortality is so high that comparatively few females survive.

Support for this interpretation was found in the Bay of Fundy, where the eastern vernal crop was still in copepodite stages in late June. If the decline from May to June is not primarily due to a depletion of the food supply or unfavorable physical conditions, excessive mortality in the Bay would at this time be limited to immigrants from the west. Figure 2 shows this to be the case, as the eastern stock apparently offsets the decline of western immigrants in the Bay, and the mean volume continued to increase through June.

TABLE V

Number of *C. finmarchicus* in various stages of development, April to September, 1932.

1932	No. stations	Method	Eggs	Nauplii	Copepodites	Adults
April	27	Net	4	1615	406	128
May	26	Net	301	3013	4138	319
June	26	Net	8432	1850	307	311
August	27	Pump	2158	882	69	21
September	12	Pump	753	7	35	4

Net: number per minute. Pump: number per cubic meter.

Mortality in maturing adults of subsequent generations in the Gulf and Bay was not as clearly evident because both the meter net and pump data indicate that comparatively few survived to reach even copepodite stages. Perhaps the greater number of predatory plankton animals in summer served to keep the numbers down. Some such explanation is necessary to account for the large crop derived from the relatively small parent stock in March–April as compared with that in May–June.

Length of Life of Different Broods

The marked decline in the relative percentage of adults to copepodites following the breeding period in June (Fig. 10*A*), in view of the fact that in relation to eggs and nauplii, copepodites showed no signifi-

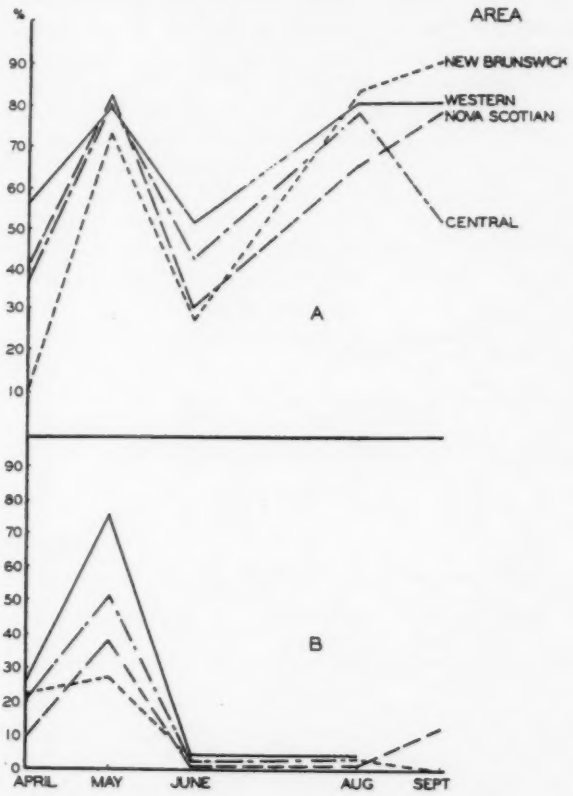

FIG. 10. *Calanus finmarchicus.* Seasonal changes in the relative percentage of (*A*) copepodites to adults, and (*B*) copepodites to eggs and nauplii.

cant increase (Fig. 10*B*), is considered to represent a dying off of adults after spawning. This appears to be a rather well-established characteristic of the species in the eastern Atlantic (Damas, 1905; Paulsen, 1906; Nicholls, 1933) and has been suggested by Bigelow (1926, p. 206) in the Gulf of Maine.

The length of life of individuals produced in early April and maturing in June would thus probably not exceed four months, allowing two and one-half months for development to maturity and a maximum of one and one-half months of adult life (spawning adults in the western region on June 28–July 1 had greatly diminished by August 11–14 in 1932). A similar period of adult life in spring and summer (one and one-half months) has been found by Nicholls in the Clyde Sea area (1933, p. 92).

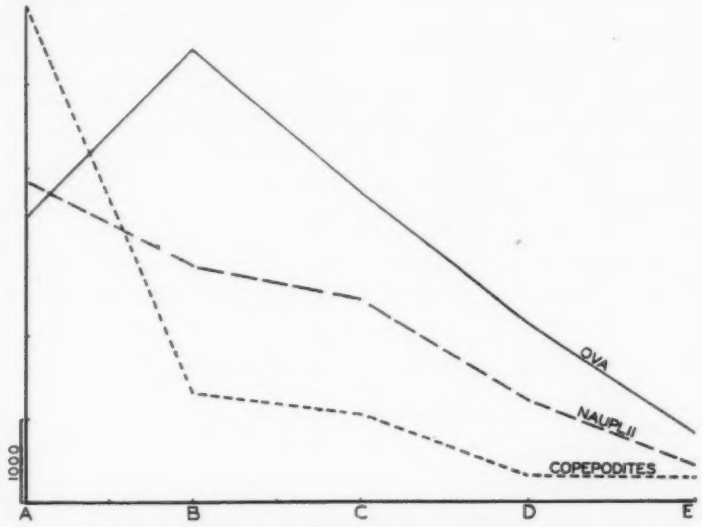

FIG. 11. Mean numbers of ova, nauplius and copepodite stages per minute of hauling for the period April–July, 1932.
Sections: *A*. Casco Bay, *B*. Penobscot Bay, *C*. Mt. Desert, *D*. Moose Peak, *E*. Cape Spencer.

In regard to the winter stock, in cold areas with fewer generations the length of life must be longer than in regions such as the Clyde Sea where spawning begins very early in the year. Our data would indicate that the increase in the number of copepodites in September, observed by Bigelow (1926, p. 207) in the Gulf and in 1932 in the drift entering the Bay of Fundy (increased from 0.3 per cent to 13.6 per cent, Fig. 10*B*), represented arrested development of at least a part of the June crop from the western region, supplemented perhaps to some extent by the July-August crop from the waters east of Mt. Desert. The small numbers of eggs of the third western brood in September do not appear adequate to account for the winter population. If the

winter group then consists largely of individuals produced in June–August and dying off after spawning the following March–April, a maximum existence of approximately 10–11 months is indicated. September-spawned *Calanus* would have a maximum existence of 7–8 months. In the Bay of Fundy the period must be even longer (p. 133). Nicholls estimates (1933, pp. 91–92) the length of life of *Calanus* in the Clyde Sea area to be about nine weeks after the winter group has moulted to stage VI, with a total existence of 7–8 months.

There is some evidence that after reaching maturity the males of *Calanus finmarchicus* die off some time before the females. Referring again to Willey's records from the Quoddy region (1921, p. 187), of the adults taken on February 23 in 1917, males formed 20 per cent and females 27 per cent. In 1932 on March 21, a month later, females formed 92.9 per cent of the *Calanus* stock and males 4.7 per cent. Damas (1905) also found males of this species rare except at the epoch of vernal reproduction.

TABLE VI

Number of *C. finmarchicus* found in different stages in the New Brunswick area, April to September. April–June, number per minute. July to September, number per cubic meter.

New Brunswick area	Station	Ova	Nauplius			Copepodite				
1932			I–II	III–IV	V–VI	I	II	III	IV	V
April 15	5									
May 4	5	17								
May 19	5			31	62					
June 20	5	7608		507	507				T	
July 30	5	710	140							
August 21	5	375	187							
September 16	11A		40							

Relative Importance of Different Spawning Areas

The contention of previous observers (Willey, 1921, p. 189; Bigelow 1926, p. 207) that the western Gulf probably forms the principal source of supply of *Calanus* for the entire region, is borne out by the present investigations. At all times the coastal region west of Mt. Desert yielded the greatest numbers of eggs, larvæ, and adults, and the rapid decline to the eastward (Fig. 11), combined with the distribution of larvæ, designate it as the probable source of the major part of the population in the outer Gulf, the central area, and the Bay of Fundy. The fluctuations in the mean volume and relative percentage of *Calanus* in the total region in 1932 paralleled the seasonal changes in the western area (Fig. 2).

No doubt a portion of the individuals produced in the eastern region, particularly near its western margin, survive because the stock in very small numbers can be traced through successive generations. However, over the greater part of the Bay of Fundy and the turbulent coastal region of the Gulf east of Petit Manan, there is no evidence of any successful production of *Calanus*, although eggs transported west from the latter area may complete their development in a more favorable environment.

In the Quoddy region (Station 5), somewhat removed from the path of the drift from the Gulf, hauls throughout the season consisted principally of eggs with relatively few nauplii. Copepodite stages were taken during the summer with a meter net but their numbers were so small that almost none were found in pump and fine net samples. The large brood produced by immigrant adults in late June had apparently entirely vanished a month later, and eggs of the eastern stock in late July and August seem to have disappeared by September 16. A similar condition was found in August and September, 1931.

Summary

1. *Calanus finmarchicus* is the most abundant pelagic animal in the Gulf of Maine, averaging 39.9 per cent of the total zoöplankton population during the period April to September, 1932, with a peak of 60.4 per cent in May. In the Bay of Fundy there was a mean of 35.5 per cent for the year 1931–32.

2. The adult stock varies markedly in abundance in different areas, the smallest numbers occurring in the Bay of Fundy and westward along the coast of Maine to Penobscot Bay. The population is everywhere at a minimum in late February and early March.

3. *Calanus* passes the winter largely in copepodite stage V, the final moult taking place in February and early March shortly before vernal augmentation.

4. An average difference of approximately one month in the time of spawning in the eastern and western parts of the region is reflected in succeeding generations after dispersal. Two breeding stocks can thus be distinguished.

5. In the western stock there are two well-defined breeding periods, March–April, and June–July, with a possible third in September, and two periods, April–May, and July–August in the eastern stock.

6. A developmental period of two and one-half months is indicated in both stocks, one month from nauplius I to copepodite I, one-half month from copepodite I to copepodite V, and one month for the final moult, maturation, and incubation of eggs.

7. Mortality is highest among maturing adults.

8. The total length of life in spring and summer does not exceed four months. The winter stock has a maximum existence of 10–11 months.

9. After reaching maturity the males die off before the females.

10. The western Gulf forms the principal source of supply of *Calanus* for the entire region. Propagation east of Mt. Desert in the inner Gulf is largely if not entirely, unsuccessful.

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THE INFLUENCE OF TEST MATERIALS ON REPRODUCTION IN PONTIGULASIA VAS (LEIDY) SCHOUTEDEN

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The test of *Pontigulasia vas* resembles that of *Difflugia* and other closely allied forms. It consists of small sand grains cemented together. These sand grains are collected by the animals before division and are formed into the new shell for one of the daughter individuals. According to Pateff (1926), Gruber in 1886 first described division in *Difflugia*. More recently work on division in these forms has been done by Zuelzer (1904), Goette (1916), Pateff (1926), and others.

While experimenting with *Pontigulasia vas*, it was found that reproduction could be prevented if the cultures were kept free of substances used in shell construction. Division here was apparently related to an inert foreign substance since the animals had plenty of food and were in good condition. It was then decided to investigate this problem further in the hope that the relationship between test materials, mitosis and division in this form could be made more clear.

The writer takes pleasure in expressing his appreciation for the interest of Dr. B. D. Reynolds, under whose direction this work was carried on.

Material and Methods

The *Pontigulasia* used in these experiments were collected from a number of ponds near Charlottesville, Virginia. After isolation and identification the animals were placed in Syracuse watch glasses with clean *Spirogyra*. These watch-glass cultures were kept in moist chambers. The *Pontigulasia* were examined daily, using the wide field binocular microscope, their number noted, and the water in the cultures changed. All water for these cultures was filtered and the pH value taken before using. During the time of the experiments the pH value ranged from 6.3 to 6.9. Where sand or other substances were used these materials were obtained as clean as possible and crushed in a mortar with water. The smaller fragments and water were then transferred to the cultures with pipettes. Observations on the *Pontigulasia* while gathering materials for shell building were made

with the 16 or 8 mm. objective of a microscope. Both photomicrographs and camera lucida drawings were made of these animals. It was found that serial sections of *Pontigulasia* provided the only satisfactory method to show the protoplasmic structures within the shell. Sectioning was made possible by removing the silica dioxide (SiO_2) of the shell particles with hydrofluoric acid (H_2F_2). The *Pontigulasia* were fixed with Schaudinn's or Bouin's fluid and placed in a solution of 70 per cent alcohol and 2 per cent hydrofluoric acid. This solution was kept in paraffin-coated dishes. Ten to fourteen days were needed to remove the silica. Stronger solutions were tried but damaged fixation in most cases. Following removal of the silica, the *Pontigulasia* were imbedded in paraffin by the usual method.

TABLE I

The culturing of *Pontigulasia* without shell materials and the effect on division. Cultures with shell materials are the controls.

Culture number	Material for shell	Number of animals at start	Time	Number of animals at finish	Number of divisions
			days		
1	None	5	17	5	0
Control	Glass	5	17	10	5
2	None	5	21	5	0
Control	Sand	5	21	18	13
3	None	3	33	3	0
Control	Sand	3	33	30	27
4	None	5	26	6	1
Control	Sand	5	10	10*	5

* This was accidentally destroyed on the tenth day.

Sectioning was done at 7 μ. For staining Heidenhain's iron hematoxylin and Delafield's hematoxylin were used.

EXPERIMENTS

The following experiments were made to test the effect of culturing *Pontigulasia vas* without shell materials. The cultures were run in pairs, one was supplied with powdered sand or glass, the other was not. Beside this difference they were as far as possible exactly alike in composition and received the same treatment during the course of the experiments. The cultures with materials for shell building were considered the controls. Table I gives the results of these experiments in condensed form.

The only negative result was in Culture No. 4 where one division apparently took place. Two explanations can be given. This division was noticed the second day after the culture was started and the animal,

which was small, might have been overlooked before. Secondly there might have been enough sand in the *Spirogyra* to furnish materials for the shell. This one negative result can hardly affect the conclusion that *Pontigulasia vas* will not divide unless materials for shell building are present.

It was then thought that perhaps mitotic division had taken place resulting in multinucleate forms similar to those produced in *Amœba proteus* by Chalkley and Daniel (1933). Sections of *Pontigulasia vas* which had been cultured without shell materials showed them to be in a normal condition. There was only one nucleus and, as far as could be determined, the cytoplasm appeared unaffected.

The rest of these *Pontigulasia* were given sand to determine if their power of reproduction had been affected. After some delay division took place. An individual from Culture 3 gave a typical reaction. This animal made no effort at first to collect shell materials but began to do so three days later. By the fourth day it had produced a normal offspring. It appears, therefore, that the power of reproduction had not been permanently affected.

During the experiments the actions of the *Pontigulasia* without shell materials were interesting. Much of the time was spent moving about on the bottom of the watch glasses without any attempt to feed. At such times the pseudopods would become ragged in outline with a wide hyaline area at the ends. This type of pseudopod is usually associated with the collection of test materials. Undoubtedly these animals would have collected sand had it been present. After a day or two of such moving about the animals would begin to feed again. At other times they would go into a quiescent state for several days before feeding.

Some work was done in an attempt to find when nuclear changes began in relation to the sand-gathering activities. A number of *Pontigulasia* were isolated for several days with plenty of food. Finely ground sand was then added and the animals began to collect it. The gathering of shell materials took from 4½ to 5 hours. The pseudopods were next withdrawn and the new shell was formed immediately in front of the mother individual. During this process a number of animals were fixed and sectioned. It was found that mitosis was not begun until the collecting process had been almost completed. Fig. 1, *a*, shows a section of a *Pontigulasia* which had been collecting shell materials for four hours at the time of fixation. Nuclear changes here are very slight. There is, however, a decided mass of chromatin in the center of the nucleus. The sand grains in this form are collected and held outside the mother shell similar to *Difflugia urceolata* as found by

Zuelzer (1904). The vacuoles in the cytoplasm protruding from the shell are pockets from which the sand grains have been dissolved.

Fig. 1, *b*, shows an animal which was fixed just after the collection of shell materials had been completed but before the new shell was formed. The nucleus in this animal is in middle prophase, showing the chromatin granules on a definite linin reticulum. At this stage the nucleus is much enlarged. This section also shows the details of the shell very clearly. The internal diaphragm can be seen with the cytoplasm flowing through it. This diaphragm is easily overlooked in the living condition and the constriction between the neck and the

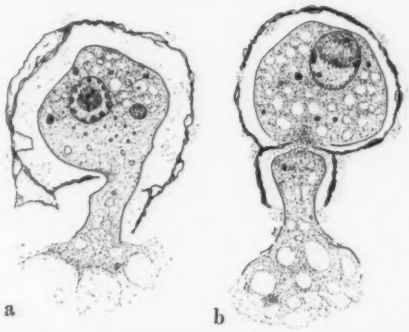

FIG. 1. Sections of *Pontigulasia vas* fixed while gathering test materials before division. Camera lucida drawings. × 400.

a. This animal had been collecting fine sand grains for 4 hours. The shell in this section was distorted when sectioned. Note that the nucleus is scarcely changed from the resting condition.

b. Section of an animal which had been collecting for 4½ hours. The formation of pseudopods had ceased but the sand grains were still a formless mass in front of the mouth of the old shell. The nucleus is much enlarged and shows a definite linin reticulum with the chromatin granules. Middle prophase. Note the constriction and the internal diaphragm in the shell.

fundus is not as deep in these forms as described by Penard (1902), Cash (1909), and Kudo (1931).

The process of division was not investigated further in this study, but it seems fairly certain that nuclear division is not started until the collecting of shell materials has been completed. A more complete study of binary fission in this form is being made.

DISCUSSION

As far as these experiments on the control of division in *Pontigulasia vas* were carried, it seems that reproduction can be prevented by withholding shell materials. It is possible that division might have

taken place if the cultures had been continued over a longer period of time. This appears unlikely, however, for in some uncontrolled cultures animals have been kept as long as 70 days without reproduction.

There still remains the possibility that individuals without shells were produced. If so, these naked animals could not have survived long for no evidences of them were seen.

No definite effect of inhibited division can be given here. There was a tendency for animals in cultures without sand to go into an inactive state immediately after attempting to collect shell materials. This inactive state lasted from one to three days. Also when such individuals were given sand there appeared to be a delay in collecting it and a delay in division afterwards. The animals resulting from such division, however, were normal as far as determined. If these variations are a result of inhibited division then they must be considered in the work done on nuclear division and its relation to shell materials. The animals used in this part of the work were kept without sand for a short time only in an effort to reduce any error from this source.

Nuclear division in *Pontigulasia vas* appears to be dependent on the shell materials in the cytoplasm rather than the collecting action of the animal itself. As the collecting process nears completion, the *Pontigulasia* seem to be in a very unstable physiological condition. If they are disturbed in any way the sand grains will be discarded and division will not take place. Pateff (1926) found the same condition in *Difflugia mammallaris* when about to reproduce.

Summary

1. *Pontigulasia vas* will not reproduce unless shell materials are present in the cultures.

2. The shell materials are collected for a period of approximately four hours immediately before division.

3. There are no changes in the nucleus until almost all the shell materials are collected. Nuclear changes are begun, however, before the formation of the new shell.

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THE WASTING DISEASE OF ZOSTERA MARINA[1]

I. A Phytological Investigation of the Diseased Plant

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At the time that this study was begun, mid-summer, 1933, an epidemic, wasting disease had almost entirely destroyed the normally dense beds of the common marine eel-grass, *Zostera marina*, along the Atlantic coasts of North America and Europe. Since the plant is a prominent member of the shallow water community, sheltering a variety of larval and small sea animals, serving substantially as food for migratory game birds, and checking erosion of the bottom, and because the leaves are used for packing, upholstering, insulating, and other commercial purposes for which they are often peculiarly desirable, the disappearance of the eel-grass became a problem of practical concern. It was recognized, too, that the extensive and persistent wasting of *Zostera* might indicate phytopathological relationships in the sea as significant as the more evident conditions in land vegetation. A further impetus was given this investigation by the popularization of a number of physical and chemical hypotheses for the depletion, of restricted application, and usually of a prejudicial nature.

In France, Holland, Denmark, and Canada investigations were already in progress (6), (7), (11), (13), (18). The history of the disease here and abroad, the current condition of the eel-grass beds, and the economic and ecological implications of the wasting have been developed by a number of observers, and comprehensive summaries have been published from time to time by the U. S. Bureau of Biological Survey and others (1), (3), (6), (7), (10), (14).

This paper is an account of investigations upon the pathology of the diseased eel-grass and upon the specific parasitic nature of an unidentified *Labyrinthula*, an amœba-like organism with mycetozoan affinities, found in the leaves of the plant over the entire range of the epidemic condition. They were begun in the attempt to extend the findings of Messrs. Fischer-Piette, Heim, and Lami of France,

[1] Submitted as a thesis in partial fulfillment of requirements for degree of Doctor of Philosophy, Rutgers University, 1935.

[2] Contribution No. 90, Woods Hole Oceanographic Institution, and Journal Series Paper of the N. J. Agr. Exp. Station.

who early reported a pathogenic bacterium as active in the diseased *Zostera* beds of their coast, and the work of Dr. H. Petersen of Copenhagen and Miss I. Mounce of Ottawa, who had studied a fungus associated with the wasting in Danish and Canadian waters, respectively (6), (11), (12). To these ends our results were negative (16). None of the bacteria isolated from diseased plants demonstrated pathogenic behavior in inoculation tests, and we failed to find any satisfactory physical association with the symptoms in leaves, stems, or roots in a variety of live specimens taken from the neighborhood of Woods Hole, Massachusetts, or in preserved material sent from many points along the American coast. From further exchange of specimens we were able to establish that though the fungus, *Ophiobolus halimus*, a new species described by Mounce and Diehl, was undoubtedly very abundant along the Canadian, Danish, and English coasts, it appeared only infrequently in diseased eel-grass taken from representative beds running from the coast of Maine southward, and that its development in these sporadic cases was incomplete (12), (17).

Nevertheless, the symptoms of the wasting disease were identical in the exchange specimens and preparations taken from many parts of the American and European coasts, and these have remained constant through the epidemic's duration.

Investigation of the Diseased Plant

The foliage of infected beds is represented by short, young, rapidly growing, green leaves, eight to twelve centimeters in length, which may not show the splotching and dark streaking characteristic of the disease; by leaves of medium development, badly discolored and spotted with brown or black; and by the wilted basal fragments of older leaves sloughed away shortly after the disintegration of the greater part of their green tissues. Usually, the spotting is confined to the leaf epidermis, but it may arise in any green tissue, develop into plaques of considerable area, or form mosaics of widely scattered, darkened cells. The streaking, due to the serial infection of the larger, longitudinally disposed mesophyll cells, is more striking; it is generally sharply bounded by the longitudinal mesophyll spaces and terminated by the lateral septa (Fig. 1). Its margins spread rapidly, from three to five centimeters a day, or at higher rates during the warm summer when the disease is most active.

Often, but not always, the cuticle and cortex of the stem are irregularly pigmented with dark brown and black; the condition is not uniformly associated with the disease. After defoliation of the plant, through local activity of the parasite in the leaves, the stem may

persist for one or two seasons to produce several new shoots each season —manifest in the apparent redevelopment of wasted beds. Eventually the reserve food supply, the accumulation of which has been limited by wasting, is exhausted and the weakened stems are invaded by bacteria, fungi, and attending saprophytes; the less vascular true roots decompose rapidly in the mud.

Microscopic examination of discolored and streaked tissue does not always reveal the protozoan parasite with which this paper is concerned; the reason for this discrepancy will be clear when its life history is described. On the other hand, the changes that can be directly observed following experimental infection of the leaf are identical with those given by histological preparations of the diseased plant from naturally infected beds. Within a few hours after the cells have been entered by the parasite, the chloroplasts become olive or straw colored and either pass to the periphery of the cell where they adhere in masses, or coalesce near the center. Soon afterward they fragment, and the small particles of chloroplast material become dispersed through the cell or coagulate into dark, irregular clots. The nucleus persists for a long time after the breakdown of the chloroplasts, but finally takes on a spherical outline, granular texture, and brown or black pigmentation; often it is hidden by debris. In addition, dark globules of resinous substances, apparently produced by the injured cell itself, and not identified with any of the broken structures, make their appearances at intervals along the cell wall.

No changes attributable to the direct attack of the parasite upon the stems and roots could be observed; though the organism was found in the cells of the stem cortex several times, it was never abundant there nor uniformly associated with the discoloration of those parts. The damage brought about by the protozoan parasite is reflected systemically only through the destruction of the plant's food-synthesizing mechanism. Death follows the loss of the photo-synthetic function of the green leaves and the exhaustion of reserve food in the stems.

Demonstration of the *Labyrinthula* in Diseased Eel-Grass

The spindle-shaped cells and interconnecting system of pseudopodia peculiar to the *Labyrinthulæ* and characteristic of the protozoan parasite of eel-grass were first observed in stained sections of diseased leaves taken from the region of Nantucket, Massachusetts, in the spring of 1934. That they had been overlooked until this time may have been due to their unusual distribution in the infected leaf, always well in advance of the visible streaking and discoloration itself, in the green tissues beyond the borders of the visible symptoms, and also

to their very slight refractility. Their staining, too, is highly specific and difficult; to date a sequence of safranin, carbol-thionin, and Orange G. dyes, though uncertain in results, has given the best demonstration in formol-acetic-alcohol preserved field specimens. In these stained preparations the spindle-shaped cells of the *Labyrinthula* occurred with such persistence and were so suggestively oriented in the pathological picture that they were singled out as being in some way related to the disease.

A careful re-examination of specimens received from many points south of Woods Hole to Beaufort, N. C., confirmed the association in those waters, and later, when specimens were exchanged with Miss Mounce in St. Andrews, N. B., and with Mr. Tutin in Plymouth, England, the relations in these regions became evident also. Not long afterward, viable *Labyrinthulæ* were demonstrated by these investigators in their respective localities. Working independently, Mr. Loraine Young observed and studied the organism in diseased leaves in returning beds near Mt. Desert Island, Maine.

Dr. Frederick S. Sparrow of Dartmouth College identified the organism as one of the *Labyrinthulæ*, a family of the *Proteomyxa*, characterized by peculiar interconnecting pseudopodia; he also made many valuable suggestions relating to the culture and manipulation of the form.

In many features the *Labyrinthula* in *Zostera* is similar to the members of the family that have been described as parasitic upon marine algæ (2), (4), (9). It is not possible to identify the species with any of these at this time, however.

As found in the infected plant, the *Labyrinthula* may appear as a number of individual, colorless, slightly refractile, spindle-shaped cells from 10 to 20 mμ long and 5 to 8 mμ in diameter, distributed at random or in unrelated clusters through the green tissues and longitudinal spaces of the mesophyll (Fig. 2); as net-like aggregates of individuals connected together by a web of fine, branching, terminally secreted pseudopodia similarly disposed in the parasitized leaf (Fig. 3); as pseudoplasmodia formed of many individual spindles closely massed into thick cords or less regular strands (Fig. 4); or as what is apparently a resting stage—cyst-like, spherical bodies, about 6 to 10 mμ in diameter, light brown in color, surrounded by less dense, thick cuticles, and occurring mixed with residues in the older infected tissues (Fig. 5).

The spindles are motile; in the net-like organization they glide along the interconnecting pseudopodial web at rates in excess of 50 mμ per minute. They tend to concentrate in newly invaded tissue entrance to which is gained by local digestion of the cell walls. The pseudo-

plasmodium, sometimes relatively massive, also performs progressive movements, though not so rapidly as the individual cells; terminal masses of spindles may disperse to form a number of more lively branches (Fig. 6).

FIG. 1 FIG. 2

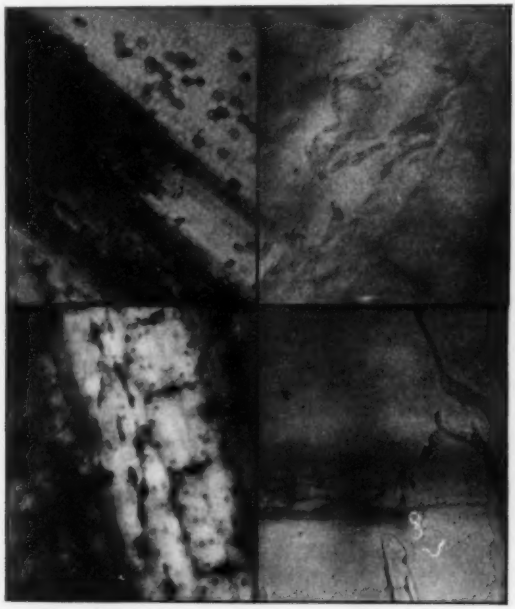

FIG. 3 FIG. 4

PLATE I

FIG. 1. Typical symptoms of the wasting disease in the leaf. Note extensive streaking in the mesophyll and mosaic infection of epidermal cells at upper right. Photomicrograph of clove oil-cleared preparation, stained with safranin—× 410.

FIG. 2. Individual spindle cells of *Labyrinthula*. No evidences here of pseudopodial net-structure. Photomicrograph from living preparation—× 480.

FIG. 3. Chain-like aggregates of connected spindles extending through longitudinal mesophyll space at boundary of infected zone. Note broken tissue at left and healthy mesophyll cells at right. Photomicrograph of living preparation—× 445.

FIG. 4. Pseudoplasmodium in recently infected mesophyll tissue. Note early stages of degeneration in invaded mesophyll cells at left. Photomicrograph of living preparation—× 480.

All of these behaviors may be observed readily in damp cell mounts of excised, living, infected leaf tissue, and they may be extended to the study of the parasitic behavior by adding fresh, uninfected tissue to

these mounts, a procedure giving an excellent demonstration of the migration of spindles and pseudoplasmodia into new host tissue.

Demonstration of Parasitism

A method of this design was employed in establishing the parasitic nature of the *Labyrinthula* found in eel-grass. Thirty-six damp cell,

Fig. 5

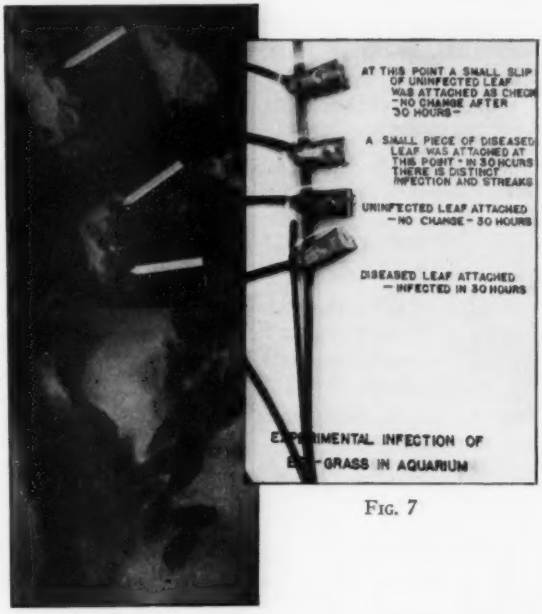
AT THIS POINT A SMALL SLIP OF UNINFECTED LEAF WAS ATTACHED AS CHECK - NO CHANGE AFTER 30 HOURS -
A SMALL PIECE OF DISEASED LEAF WAS ATTACHED AT THIS POINT - IN 30 HOURS THERE IS DISTINCT INFECTION AND STREAKS
UNINFECTED LEAF ATTACHED - NO CHANGE - 30 HOURS
DISEASED LEAF ATTACHED - INFECTED IN 30 HOURS
IMENTAL INFECTION OF
GRASS IN AQUARIUM

Fig. 7

Fig. 6

Plate II

Fig. 5. Cyst-like resting stages of the *Labyrinthula* lying in mesophyll spaces of blackened leaf—× 475.

Fig. 6. Dissolution of pseudoplasmodium into individual spindles. Here the centrally placed "vacuoles" may be distinguished. Photomicrograph of living preparation—× 1520.

Fig. 7. Demonstration of inoculation method. This leaf was taken from an aquarium experiment. In the field the inocula and checks were spaced at intervals of about four inches. Here the tendency of inocula to overrun checks is already evident.

hanging drop preparations were made, each consisting of small tangential sections of living, uninfected leaf held against the under side of the cover-glass by a thin droplet of Seitz-filtered sea water in which, at

a distance of about a millimeter from the test tissue, small pieces of diseased leaf containing viable *Labyrinthula* cells were also placed. Care was taken to avoid serious contamination.

Within eight to forty-eight hours the migration of motile spindles or the extension of pseudoplasmodia from diseased sections to the originally uninfested tissue and the invasion of the new host was evident in these preparations.

In these recently invaded pieces of host tissue the progress of disintegration was identical to that observed in sections of the living, diseased leaf studied earlier. Thirty of these preparations gave positive results, while ten check mounts containing green tissue and no "inocula" demonstrated that the tissue might be maintained in good health under the conditions of the experiment. One of these checks became contaminated by an air fungus, but nowhere else in these tests did mycelium appear.

Field Inoculation Experiments

That damp cell inoculation of excised leaf tissue may not indicate the parasitic habit of the *Labyrinthula* in nature is quite clear, but this criticism is effectively answered in field experiments repeated a number of times in selected eel-grass beds in Hadley's Harbor, near Woods Hole, during the summers 1934 and 1935.

Here healthy plants were inoculated by fastening to their leaves small pieces of diseased leaf, and check preparations were made by attaching pieces of normal leaf, or bits of paper to the same leaves. These were usually arranged alternately as shown in Fig. 7. Small cork clamps held the inocula and checks in place and great care was exercised to avoid mechanical injury of the test plant. The experiments during late summer, 1934, were directed toward determining the validity of this technique and adapting it to the more detailed studies that followed, but it was notable in these preliminary trials, repeated four times in two separate beds, and using from twenty to twenty-five tests in each trial, that the plants uniformly showed a degree of infection greater than 50 per cent at the end of two days exposure. The inoculated leaves were always marked with the symptoms of the disease generating from the points at which the inocula were attached, and the checks were free from any discoloration. Sections prepared from the newly infected leaves invariably showed heavy invasions of viable *Labyrinthulæ*, and of no other form, and such sections attached to clean plants brought about infection in the new host.

During the following summer, 1935, the field inoculation experiments were resumed in more detail to confirm the impressions of the

previous year and to discover why the *Ophiobolus* did not appear in the inoculated leaves, though the mycelium had been found occasionally in specimens taken from nearby beds. The same method of inoculation was followed; about sixty inoculations and checks were prepared each time, but instead of removing all of the plants at the end of two days as before, part were removed and examined after one day, another aliquot after two days, and the remainder after three days.

These specimens were studied very closely; free-hand sections were prepared from both checks and inoculations and inspected under the high dry powers of the microscope. Table I summarizes the results of this search.

TABLE I

Incidence of Labyrinthula and other Invaders in Field Inoculation Experiments on Zostera marina

	Specimens taken after		
	24 hrs.	2 days	3 days
Number of inoculations	14	13	25
Number of inoculations showing symptoms of disease	8	9	22
Number of inoculations showing invasion by *Labyrinthula*	7	9	22
Number of inoculations showing invasion by bacteria or fungi	0	0	3‡
Number of checks	14	9	22
Number of checks showing streaks or other symptoms	0	3*	12†
Number of checks showing invasion by *Labyrinthula*	0	2*	11†
Number of checks showing invasion by bacteria or fungi	0	0	0

* Two of these checks were obviously overrun by extensive streaking arising from the inoculated region of the same leaf. The third showed some slight disintegration but no invaders—perhaps mechanical injury.

† In ten cases there was evident overrunning of the checks by extension of streaking from the point of inoculation. One is a case of spontaneous infection, clearly, because the inoculation in that case did not "take."

‡ The form appeared to be a filamentous fungus, but the mycelium was very poorly developed and mixed in with *Labyrinthula* and debris.

Later in the summer the experiments were repeated with some modifications that were designed to cut down the tendency of inoculations to overrun the check areas. These changes were not successful,

but the results obtained confirm those secured earlier in the season and are given in Table II.

TABLE II

Incidence of Labyrinthula and other Invaders in Field Inoculation Experiments on Zostera marina

	Specimens taken after -		
	24 hrs.	2 days	3 days
Number of inoculations	14	13	24
Number of inoculations showing symptoms of disease when removed	9	9	21
Number of inoculations showing invasion by *Labyrinthula*	10§	11§	22
Number of inoculations showing invasion by bacteria or fungi	0	0	3‡
Number of checks	14	10	22
Number of checks showing streaks or other symptoms	0	3*	12†
Number of checks showing invasion by *Labyrinthula*	0	3*	12†
Number of checks showing invasion by bacteria or fungi	0	0	0

* Despite wider separation of checks and inoculations the three checks were in these cases completely overrun by the development of streaking from the point of inoculation.

† In nine cases there was obvious overrunning of the checks. In two cases the check had become infected and had inoculated the leaf to which it was attached; in the other, the leaf had become infected and had inoculated the check.

‡ Fine mycelium in two cases; in the other distinctly heavy development of thick-walled mycelium.

§ Indicating infection so recent that the recognizable symptoms of darkening have not yet developed.

CULTURE OF THE *Labyrinthula*

A number of unsuccessful attempts have been made to transfer the *Labyrinthula* from its host to artificial media of various types. We have also tried species of *Bryopsis* and *Cladophora* as substitute hosts, choices determined by previously reported parasitism of these algæ by *Labyrinthulæ* (4).

Large numbers of spindles and masses of pseudoplasmodia free of obscuring host tissue could be obtained for morphological study, however, by culturing small pieces of infected leaf in sealed, hanging drop mounts. When these were exposed to diffused daylight for a few hours at room temperatures, the spindles migrated to the periphery of the supporting droplet in cord-like aggregates, sprays, and clusters.

If desired, the cover-slip could be lifted from the slide and the host tissue removed with fine tweezers to leave an unobstructed field in which the movement and development of the *Labyrinthula* might be studied over a period of four to eight hours.

Some cursory inoculation experiments using the isolated *Labyrinthulæ* as inocula have not given satisfactory results.

Discussion and Summary

From distribution studies on specimens representing the range of the wasting disease and taken over a period of three years, it appears that the *Labyrinthula* demonstrated in these experiments is universally associated with the epidemic condition. It did not occur, for example in eel-grass from Nanaimo, B. C., where the wasting disease has not been active, though the leaves of the plants were marked by lesions of various bacteria, fungi, and worms.

Histological examination and inoculation experiments indicate the true parasitic nature of the *Labyrinthula*. The rapidity with which it produces infection in these experiments is commensural with the aggressive habit of the disease in nature, and in this respect it seems to be the only organism transferred in a time interval comparable with the severity of the disease. The appearance of mycelium after several days, following the invasion by *Labyrinthulæ*, suggests a minor rôle for the fungus. Until this is definitely established by inoculation tests in the more northern waters the alternative of two active parasites must be considered. This does not seem likely, however, for the *Labyrinthula* is present and viable in the middle Atlantic regions throughout the year—it is notably more destructive in the late summer. If the *Ophiobolus* be a true parasite, its parasitic habit must be restricted to the Canadian and north European waters where it reaches its complete development.

From its observed behavior the *Labyrinthula* appears to be a specific parasite of *Zostera marina* L., but until the life history of the organism has been studied more carefully, it seems unwise to constitute a new species. It will be necessary, too, to attempt further host transfer experiments and to search for the *Labyrinthula* among related plants and members of the same community.

No attempt has been made in this paper to consider pertinent epidemiological problems; these are reserved for a report on field studies now in progress.

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WOODS HOLE, MASS.

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For a number of years the Supply Department has been furnishing Living Marine Materials. From experience it has been found that during the period between the first of November and the end of February these live animals and plants can be shipped with the most success. This service has proven to be of great value to both instructor and student, and we have had numerous letters of commendation on our Live Marine Material. We would be very glad to have the opportunity of sending one of these sets to your school or college. Instructions regarding these shipments will be gladly sent upon request, and shipments are made from November through February with the guarantee that they will ship in good condition as far west as the Mississippi and as far south as Georgia.

Regarding the Dogfish material, we believe we can state without contradiction that those specimens prepared by the Marine Biological Supply Department are superior to any on the market. The long experience of the preparators and the excellent boat and laboratory equipment enable us to handle large numbers of Dogfish during the period when they are migrating, and we have a complete stock of all sizes of plain and injected spiny Dogfish, heads for brain study and specimens in brine for skeletons.

All materials purchased from us are guaranteed to give satisfaction, and any items which are found to be otherwise may be returned for full credit at our expense.

Catalogues on request

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